COMPOSITION AND STABILITY OF PHYTOCHEMICALS DURING FOOD PROCESSING

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Dietary phytochemicals are thought to reduce the incidence of chronic degenerative diseases. The concentration of these phytochemicals has been extensively studied, but less is known about their stability during food processing. The objective of this study was to determine the stability of lignans and other phytochemicals during the vinification and brewing processes. The amount of secoisolariciresinol diglucoside (SDG), gallic acid, caffeic acid, coumaric acid, chlorogenic acid, and ferulic acid increased up to 45 % during the vinification process; however, the amount of folic acid remained unchanged. SDG content was determined in barley for the first time. The SDG content also varied among barley varieties and showed a year-to-year variation. In addition, no SDG was detected during the mashing, lautering, boiling, and fermentation steps of the brewing process. Overall, processing techniques used in this study caused various effects on the stability of phytochemicals.

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1. INTRODUCTION

The consumption of foods that are rich in phytochemicals may reduce the incidence of birth defects, chronic degenerative diseases, including cancer, cardiovascular, and neurodegenerative diseases (Blancquaert et al 2010; Liu 2013). Foods that contain these phytochemicals include whole-grain cereals (e.g. wheat and barley), oilseeds (e.g. sesame and flaxseed), legumes, fruits, and vegetables (Milder et al 2005). For instance, the health benefits associated with flaxseed consumption have been recognized for centuries (Klimaszewski 2000).

Flaxseed, an ancient dicotyledonous crop, contains functional ingredients that promote health (Liu 2013). One such ingredient is lignan, a di-phenolic compound with a 2, 3-dibenzylbutane skeleton structure (Madhusudhan et al 2000; Setchell et al 1980). Flaxseed is by far the richest known source of the lignan secoisolariciresinol (SECO), which can also exist in the form of secoisolariciresinol diglucoside (SDG) after glycosylation (i.e. the attachment of two glucose molecules on SECO) (Ford et al 2001). In the intestinal tract, these plant lignans are converted to mammalian lignans, making them biologically active, and the increased intake of these lignans has been correlated with reduced hormone-associated cancers (Wang 2002).

Apart from lignans, flaxseed contains other bioactive compounds, including vitamins (e.g. folates) and phenolic acids (e.g. ferulic acid, gallic acid, m-coumaric acid, caffeic acid, and chlorogenic acid) (Liu 2013). Similarly, these compounds are believed to reduce the incidence of chronic diseases. The incorporation of folate in the diet, for example, lowers the risk of neural tube birth defects (NTDs), cardiovascular diseases, and megaloblastic anemia (Blancquaert et al 2010). Due to its potential as a functional food, flaxseed has been incorporated into baked goods, including breads and pastas for its lignan, as well as for other essential nutrients (Durazzo et al 2013; Muir and Westcott 2000).

To ensure the inclusion of such phytochemicals in our diet, the affect of food processing on bioavailability of bioactive compounds is essential. For instance, the milling of spices and nuts enhances the loss of volatile compounds due to the temperature increase during milling (Fellows 2000). The amount of vitamin C decreased, by 78 %, after slicing (size-reduction) cucumbers (Erdman and Erdman 1982). In addition, the blanching process can inactivate enzymes and reduce the amount of ascorbic acid (Fellows 2000). Pasteurization, a relatively mild process, is responsible for the reduction of serum proteins and vitamins (Fellows 2000). During sterilization, 10-20 % loss of amino acids in canned meat has been observed. Extrusion process also reduces (up to 50 %) the amount of ascorbic acid and β-carotene in cereals (Harper 1979). Microbes, used during fermentation processes, also are known to change the composition of proteins, fats, and carbohydrates (Dworschak 1982).

Many studies have been conducted on the concentration of bioactive compounds; however, little is known about their stability during food processing. Researchers also have analyzed the lignan stability during the production of bakery and dairy products (Liukkonen et al 2003; Hyvarinen et al 2006a, b). However, studies remain scarce on other processing techniques, including malting, milling, vinification, and brewing. In this study, the stability of SDG and other phytochemicals will be determined during fermentation and aging processes. In addition, the effect of variety, malting, and brewing on SDG content were investigated.

2. LITERATURE REVIEW

2.1. Introduction to flaxseed

Flax (*Linum usitatissimum L.*) is an ancient plant grown for its seed to produce oil and meal, as well as for its stem to produce fiber (Berglund and Zollinger 2007). Flax oil is very susceptible to oxidation, making it desirable for industrial purposes, including paints, varnishes, and printing inks (Daun et al 2003; Ehrensing 2008). For thousands of years, flaxseed has been consumed by humans, but ever since the industrial revolution, flax oil has been predominantly used for industrial purposes (Daun et al 2003). However, flax oil is considered healthy and is often sold in health food stores. The ground flax meal, on the other hand, is used for animal feed and also added to bakery products to enhance nutrition. Furthermore, the flax fiber is used to make fine linen cloth and paper (Oplinger et al 1989).

Canada is the largest producer and exporter of flaxseed, accounting for 40 % of the world's production. In the United States, the four major states that produce flaxseed include North Dakota (95.1 %), South Dakota (2.1 %), Montana (2.0 %) and Minnesota (0.8 %) (NASS 2013). Although flaxseed, currently, is used for the production of flaxseed oil, paint, and textile fiber, its use for animal feed and human consumption is growing (Laurence and Mike 2014)

2.2. History on flaxseed

The consumption of flaxseed dates back to 5000 BC. Historians have documented the use of flaxseed as medicinal ingredients. For example, Egyptians used to carry flaxseeds in their bag for medicinal purposes (Klimaszewski 2000). In addition, Egyptians used the stem of the plant to make linen cloth for wrapping mummies (Klimaszewski 2000; Anonymous 2007). In the 8th Century, King Charlemagne of France mandated the consumption of flaxseed after recognizing the health benefits associated with it (Anonymous 2007). Hippocrates, the father of modern

medicine, also recognized the use of flaxseed as a laxative and poultice (Anonymous 2007). The US National Cancer Institute has recognized flaxseed as a potential food ingredient for providing basic nutrition and protection from cancer and coronary heart disease (Carter 1993). Health benefits associated with flaxseed consumption have been well acknowledged for centuries, and many ongoing studies are being conducted to further identify its health benefits (Muir and Westcott 2003).

2.3. Flaxseed composition

2.3.1. Major chemical constituents

The health benefits of flaxseed relate to the seed composition. Flaxseed is primarily composed of lipids (40 %), dietary fiber (30 %), protein (20 %), and ash (4 %), which are located separately in different parts of the seed (Daun et al 2003). The composition can also vary depending on the variety of the flaxseed and growing environment (Daun et al 2003).

2.3.1.1. Lipid

Flaxseed structure is composed of cotyledon (55 %), seed coat and hull (36 %), and embryo (4 %). The cotyledon is the major storage tissue for oil (Daun et al 2003). The lipid constituent or oil mainly exists in triacylglyceride (98 %), phospholipids (0.9 %), and free fatty acid (0.1 %) forms (Daun et al 2003). In oilseeds, the oil that is extracted via non-polar lipid solvent (e.g. hexane) is a neutral lipid (e.g. triacylglyceride) (Daun et al 2003). On the other hand, the lipids that are not extracted through non-polar solvents are polar lipids; to extract these lipids, polar solvents or mixtures of solvents (e.g. chloroform and methanol) must be used. The seed is also recognized for its high content of α-linolenic acid (ALA), which makes up more than 50 % of the total fatty acid composition of the lipid (Oomah 2003). In addition, the following fatty acids are found in moderate amounts: palmitic (~ 5 %), stearic (~ 3 %), oleic (~ 18 %), and

linoleic (~ 14 %). According to Eckey (1954), the high degree of unsaturation in oils is positively related to ALA content, and negatively associated with the saturated fatty acids (palmitic and stearic) and unsaturated fatty acids (i.e. oleic and linoleic).

2.3.1.2. Carbohydrate

In flaxseed, digestible carbohydrates (e.g. simple sugars and starch) are present in small amounts (Daun et al 2003). The majority (28 % of dry flaxseed weight) of these consists of non-digestible carbohydrates (i.e. dietary fiber), which are not broken down by human digestive enzymes (Daun et al 2003). Flaxseed is also rich in soluble (e.g. gums and pectin) and insoluble (e.g. cellulose and lignin) dietary fibers (Rubilar et al 2010). The application of various extraction methods and chemical analysis affects the ratio of these two dietary fibers (soluble: insoluble), which can vary from 20:80 (%) to 40:60 (%) (Daun et al 2003). The hull or more specifically the outer most layer contains the soluble fiber, also referred to as mucilage (Mazza and Biliaderis 1989). Approximately 8 % of flaxseed weight is mucilage, and its content depends on cultivar and extraction methods (Oomah et al 1995; Cui et al 1996). From the food industry perspective, mucilage can be used as a food gum due to its thickening and emulsifying properties. From the health perspective, mucilage is associated with lowering cholesterol content in the blood stream (Daun et al 2003).

2.3.1.3. Protein

Flaxseed contains approximately 23 % crude protein (i.e. nitrogen both from protein and non-protein sources) or 20 % true protein (i.e. nitrogen only from protein sources) (Daun et al 2003). Flaxseed also contains two major proteins, namely globulin (80 %) and albumin (20 %), but the quality of the total protein depends on the adequate presence of three essential limiting amino acids; the first being lysine, then methionine and cysteine (Bhatty 1995; Sammour 1999).

In flaxseed, like other oilseeds, inverse association between oil and protein content has been reported (Daun et al 2003).

2.3.2. Minor chemical constituents

2.3.2.1. Phenols in flaxseed

Apart from the major constituents, flaxseeds contain phenolic compounds (phenols) that are involved in the plant's reproduction, growth, and defense mechanism against diseases (Dai and Mumper 2010). Common phenols found in flaxseed consist of a hydroxyl group (one or more) attached directly to an aromatic ring (one or more). Phenols protect plants against parasites and pathogens, and are also responsible for the pigmentation of fruits and vegetables (e.g. apples and beets). Identified phenols in flaxseed include phenolic acids (e.g. p-coumaric, ferulic and caffeic acid) and flavonoids (Oomah et al 1995).

2.3.2.1.1. Phenolic Acids

Flaxseed contains approximately 8-10 g/kg total phenolic acids. The seed also contains both esterified (5 g/ kg) and etherified phenolic acids (3-5 g/kg). Phenolic acids are derivatives of benzoic and cinnamic acid (Oomah et al 1995). In plants, they are products of secondary metabolism and categorized into phenolic acids, flavonoids, stilbenes, coumarins, and tannins (Liu 2004). In our diet, phenolic acids account for up to one-third of the phenolics and the remaining, two-third, comes from flavonoids (Liu 2004). Phenolic acids are grouped into two groups, namely, hydroxybenzoic acid (e.g. p-hydroxybenzoic and gallic) and hydroxycinnamic acid (e.g. p-coumaric, caffeic, chlorogenic, and ferulic acids) derivatives. In foods, the hydroxybenzoic acids are not found in their free form; instead, they are found in complexes with other plant components, such as lignin, hydrolyzable tannins, fiber, sugar, and proteins (Liu 2004). Similarly, the hydroxycinnamic acids are found in the bound form or linked with

cellulose, lignin, and proteins via ester bonds (Liu 2004). During food processing, some bound phenolic acids become free. For instance, bound ferulic acid becomes free after thermal processing, pasteurization, fermentation, and freezing (Dewanto et al 2002).

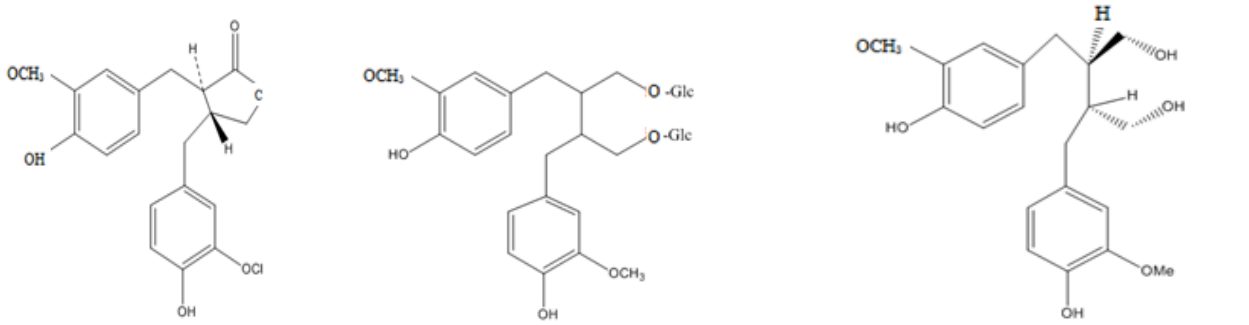
2.3.2.1.2. Flavonoids

Another common phenol in flaxseed includes flavonoid, a polyphenol made up of C6-C3-C6 skeleton with two aromatic rings attached via three-carbon bridge (Oomah et al 1996). Some examples of flavonoids include anthocyanins, flavanols, flavones, flavanones, and flavonols. Flaxseed contains approximately 0.3-0.71 g/kg total flavonoids and this amount can vary depending on environmental conditions (e.g. cultivar) (Oomah et al 1996). Furthermore, flavonoids in flaxseed exist in the form of glucosides, including herbacetin 3, 8-O-diglucopyranoside, herbacetin 3, 7-O-dimethyl ether, and kaempferol 3, and 7-O-diglucopyranoside (Qiu et al 1999). In addition, some of these glucosides (e.g. herbacetin diglucoside-HDG) can be found linked to lignan macromolecule through 3-hydroxy-3-methylglutaric acid (HMGA) (Struijus et al 2007).

2.3.2.2. Lignans in flaxseed

Other than the common phenols, flaxseed contains a di-phenolic compound with a 2, 3-dibenzylbutane skeleton structure called lignans (Madhusudhan et al 2000; Setchell et al 1980). Lignans (Figure 1) are composed of two coniferyl alcohol residues that are found in the plant cell wall (Jenab et al 1999; Muir and Westcott, 2003). Different types of lignans exist, but secoisolariciresinol (SECO) is one type of lignan that is present in large quantities. Other types of lignans found in small quantity, include matairesinol, lariciresinol, 7-hydroxymatairesinol, shonanin (3, 4-divanillyltetrahydrofuran) and pinoresinol diglucoside (Heinonen et al 2001; Liggins et al 2000). The major lignan, SECO, can also exist as secoisolariciresinol diglucoside

(SDG) after the attachment of two glucose molecules on SECO via glycosylation (Ford et al 2001).



Matairesinol (Secoisolariciresinol diglucoside) SDG (Secoisolariciresinol) SECO

Figure 1. Example of three commonly known plant lignan structures (Adapted from Muir and Westcott 2003).

In flaxseed, SDG is concentrated in the seed coat and accounts for 0.74 −1.9 % of the seed weight (Muir 2006; Oomah and Sitter 2009). In flaxseed, SDG is not found in its free form; instead it exists as a macromolecule. This macromolecule or lignan complex contains oligomers of SDG residues connected with 3-hydroxy-3-methylglutaric acid (HMGA). Five SDG residues (with an average molecular mass of 4000) have been identified in the lignan complex (Kamal-Eldin et al 2001). In addition to SDG, coumaric acid glucoside (CouAG) and ferulic acid glucoside (FeAG), along with HMGA, have been identified (Ford et al 2001; Johnsson et al 2002). To obtain free SDG, solvent treatment with basic ethanol or methanol is used. Furthermore, free SDG can further be metabolized, via acid hydrolysis, into secoisolariciresinol (SECO), where the two glucose molecules are removed (Toure and Xueming 2010).

2.3.2.3. Sources of lignans

Plants that contain lignan include whole-grain cereals, such as wheat, oats and barley; oilseeds, such as sesame seed, sunflower seed, and flaxseed; legumes, such as beans, lentils, and soybeans; fruits, such as strawberry, kiwi, and apricot; and vegetables, such as broccoli, carrots,

and garlic. Of these plants, oilseeds, particularly flaxseeds (Table 1), are by far the richest known source of plant lignan (Milder et al 2005; Toure and Xueming 2010).

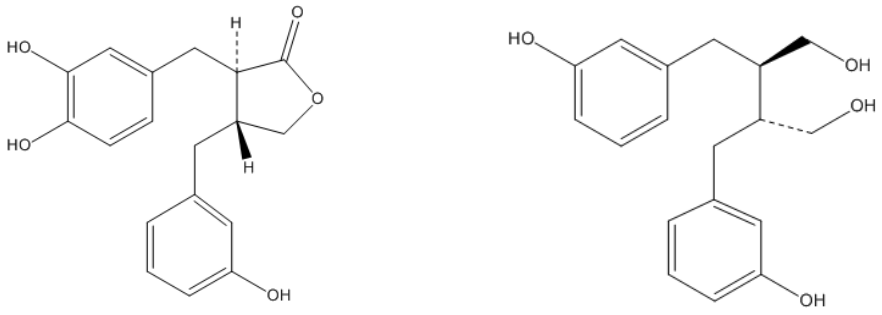
Table 1. Lignan (SECO) content (µg/100g dw) of selected plant foods.

Plant foods	SECO (µg/100g)	Source
Flaxseed	369900	Mazur et al (1996)
Barley	58	Mazur and Adlercreutz (1998)
Broccoli	414	Mazur and Adlercreutz (1998)
Wheat	8.1-868	Adlercreutz and Mazur (1998); Smeds et al (2007)
Guava	700	Mazur (1998)

2.3.2.4. Lignans in mammals

Bakke and Klosterman (1956) were the first to isolate and identify SDG from flaxseed; however, no biological activity of the lignan (SDG) was reported. Approximately 20 years later, researchers conducting a hormone study detected two unknown compounds in urine that had great similarity to the structure of plant lignans. The unknowns were later identified as enterolactone (ENL) and enterodiol (ED), collectively known as the mammalian lignans (Wang 2002). Setchell (1995) proposed that the origin of these mammalian lignans were due to bacteria in the intestinal tract converting the plant lignans into mammalian lignans (Figure 2). First, the gastrointestinal (GI) bacteria act upon SDG to release the carbohydrate free lignan, SECO (Toure and Xueming 2010). With the help of colonic bacteria, SECO further undergoes dehydroxylation and demethylation, resulting in the mammalian lignan– enterodiol (ED). The ED is oxidized via GI microbial flora, forming enterolactone (ENL). In the past, the two plant lignans (matairesinol and SDG) were thought to be the only plant precursors for the formation of the two mammalian

lignans, ED and EL. However, other lignans, including lariciresinol, 7-hydroxymatairesinol, and pinoresinol diglucoside have been identified as precursors for the two mammalian lignans (Heinonen et al 2001).



Enterolactone (ENL) Enterodiol (ED)

Figure 2. Example of two commonly known mammalian lignan structures (Adapted from Muir and Westcott 2003).

2.3.2.5. Biological activity of lignans

The mammalian lignan, ENL, has been detected in the urine of humans, rats, baboons, and vervet monkeys, where higher amounts being detected during luteal phase and early pregnancy (Wang 2002). This varying amount at different stages has led researchers to associate lignan with some biological role within the body. For instance, the stereo-chemical similarity between lignans (phenyl ring) and estrogens has led scientists to suspect lignan as having some estrogen-related activity (Wang 2002). Such plant-derived estrogens (i.e. phytoestrogens) as lignan compete with estradiol (i.e. natural estrogen hormone) and bind to estrogen receptors (Wang 2002; Figure 3). However, this binding is not as strong as the link between estradiol and the estrogen receptors, which can give lignan either an estrogenic or anti-estrogenic role. According to Dehennin et al (1982), the estrogenic role of ENL was disproved after treating mouse uterine with synthetic ENL, which ended up showing no significant change on the weight

of the uterine. On the other hand, the natural estrogen hormone (i.e. estradiol) brought some change to the weight of the uterine. Waters and Knowler (1982) concluded that an anti-estrogenic activity was related to depressed RNA synthesis. Nesbitt and Thompson (1999) conducted a study where they supplemented pre-menopausal women with breakfast meals containing 5, 15, and 25 g ground flaxseed. They analyzed the urine of their patients and observed a linear increase in mammalian lignans as the dose of flaxseed increased. Although studies on humans have been completed, caution should be taken when consuming lignans or flaxseeds during hormone-dependent periods, including pregnancy and lactation (Thompson 1998).

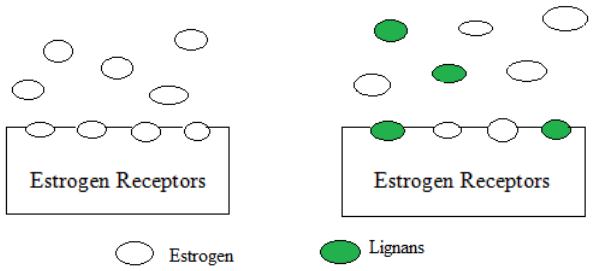


Figure 3. Lignans competing with estradiol to bind with estrogen receptors (Adapted from Mathern 2005).

2.3.2.6. Lignan incorporation in foods

In the past two decades, the inclusion of flaxseed in the diet has increased world-wide (Carraro et al 2012). Traditionally, ground and whole flaxseed has been added into baked goods, including breads and pastas for lignan fortification, as well as for other essential nutrients (Durazzo et al 2013; Muir and Westcott 2000). Although lignan-rich foods, such as flaxseed can lower breast and colon cancer risk, the introduction of flaxseed into food products has limitations (Carraro et al 2012). For instance, overtime, flaxseed can affect the flavor of the food. Flaxseed is well known for its high lipid and ALA contents, which increases flaxseed's susceptibility to

lipid oxidation (Oomah 2003). Another issue with the use of flaxseed is that it contains harmful substances such as cyanogenic glycosides and cadmium. When cyanogenic glycosides comes in contact with water, they release hydrogen cyanide, a toxic substance that can cause headaches, tachycardia (increased heart beat), and disturbance to the central nervous system (Tarpila et al 2005). Cadmium, on the other hand, causes vomiting, diarrhea, kidney disease, fragile bones, and it can be a possible cancer-causing agent (CDC 2009). To overcome possible limitations of flaxseed, SDG from flaxseeds are being extracted and directly added into bakery products, including breads and muffins, and dairy products, such as milk, cheese, yogurt, and whey drinks (Hyvarinen et al 2006a, b).

2.3.2.7. Lignan extraction

A number of extraction methods have been developed. These methods have targeted the removal of the lignan macromolecule or specific lignans. Solvent combination (e.g. ethanol: dioxane) to remove hydroxymethyl glutaric acid (HMGA) from the SDG macromolecule of a defatted flaxseed flour is one example (Klosterman and Smith 1954). Further treatment with alkaline hydrolysis degrades the remaining macromolecule of the flaxseed flour, releasing SDG (Klosterman et al 1955; Bakke and Klosterman 1956). In addition, the aglycone, i.e. SECO, can be obtained either by enzyme or acid hydrolysis of SDG. For instance, Thompson et al (1991) were able to hydrolyze the glycosidic bond through *in vitro* fermentation of gut bacteria. Mazur et al (1996), on the other hand, utilized both enzyme and acid hydrolysis in a step-wise manner. First and foremost, sample extracts are prepared using organic solvents. Then, the food extract is treated with enzyme to remove glucose molecules (i.e. hydrolysis of the glycosidic bond from SDG). This hydrolysis cannot be achieved with enzyme only; thus, the food extract is further treated with hot acid to remove any remaining glycosidic bonds (Mazur et al 1996). Employing

similar methods, Liggins et al (2000) retrieved not only SDG, but also other type of lignan called shonanin. The most common approach involves extraction of lignan macromolecule, first, using methanol/water (70:30 v/v), followed by alkaline treatment (i.e. hydrolyzes HMGA) to release the SDG free from the lignan macromolecule and acid to neutralize the previously added base (Muir and Westcott 2000; Milder et al 2004).

Muir and Westcott (2000) quantified the SDG content of baked goods that contained flaxseed or flax meal using high performance liquid chromatography (HPLC). They detected no SDG in white and specialty breads (Muir and Westcott 2000). Only the flax-containing breads contained SDG. These alkaline solvents released SDG from ester-linked lignan macromolecule. In addition, Muir and Westcott (2000) reported the effect of particle size on the recovery of SDG: finely ground flax bread facilitates the extraction of more SDG than the unground bread.

2.3.2.8. Lignan analysis

Once extracted, lignans are subjected to multiple separation and detection techniques, including reversed-phase high performance liquid chromatography (RP-HPLC), gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry (LC-MS). For foods with high lignan concentration such as flaxseed, HPLC with ultraviolet detection can be used (Milder et al 2004). HPLC-UV is less specific and sensitive than the other techniques, making it hard to analyze foods with low lignan content. HPLC with coulometric electrode array detection, on the other hand, is sensitive, but not specific enough. The GC-MS technique, on the other hand, requires extracted compounds to undergo derivitization before analysis, a technique that is time-consuming and complicated (Milder et al 2004; Wang 2002). The high specificity, sensitivity, and the absence of derivitization has made LC-MS more desirable than any of the previously mentioned techniques (Milder et al 2004).

2.3.2.8.1. HPLC analysis

The use of HPLC for analyzing SDG in flaxseed is a traditional approach developed by Westcott and Muir (1996). They were the first to develop an HPLC technique that could rapidly detect and quantify SDG in flaxseed and flaxseed meal. The following HPLC parameters were used by Westcott and Muir (1996): Symmetry C18 column (5 µm, 250 mm × 4.6 mm), mobile phase consisting of 1 % aqueous acetic acid (solvent A) and 100 % methanol (solvent B), following gradient conditions that include: A/B (v/v): 0 min (95:5), 44 min (40:60), 48 min (40:60), and 55min (95:5), and detection at 280 nm. Similarly, other phenolics, including p-coumaric acid and ferulic acid can be quantified using similar parameters (Eliasson et al 2003).

2.3.2.8.2. LC-MS analysis

Detecting lignans in flaxseeds has also been carried out using LC-MS. For instance, the following parameters were used by Popova et al (2009): A Zorbax Agilent Eclipse XDB-C18 Extend with a guard column (150 mm × 4.6 mm, 5 µm) with a temperature of 40 ^{0}C was used to separate lignans. In addition, the mobile phase consisted of solvent A (0.05 mmol L^{-1} ammonium acetate in water) and solvent B (0.05 mmol L^{-1} ammonium acetate in acetonitrile). The Gradient was set up for 22 minutes and was applied as follows: 5 min, 10 % B, and 5 – 50 min, 95 % B. The flow rate was 0.4 ml/min and the injection volume was 4-40 µL (Popova et al 2009). Popova et al (2009) also used HPLC/MS/MS analysis for lignan quantification. An Agilent 1100 HPLC coupled with API 3000 triple-quadrupole and an MS system with a turbo-ion spray was used. The following parameters were optimized: temperature of ionization (400^{0}C), and nebulizer gas (air) flow rate (14 L/min). The same solvent system and gradient was used as described above.

2.3.2.8.3. Other analysis techniques

Multiple detection techniques have also been employed to analyze the mammalian lignans in urine and plasma. The GC technique is less desirable as it requires derivitization before analysis, making it tedious and complicated (Wang 2002). An alternative to this technique was to couple the GC with ion mobility spectrometry (IMS). The IMS helps in the separation of ions based upon their charge, mass, and collisional cross-sectional area. The ions are identified based on the migration time or the time it takes the ions to reach a collector electrode (Wang 2002). Often, this GC-IMS technique requires chromatographic separation beforehand due to its low-resolution power of detecting complex biological samples (e.g. urine and plasma). Furthermore, an HPLC coupled with coulometric electrode array detection is more sensitive than UV or diode array detection, but less sensitive than the GC-IMS. Unlike food lignan analysis, the GC-IMS is a more preferred analytical technique for detecting mammalian lignans (Wang 2002).

2.3.2.9. Stability of lignan during food processing

The stability of SDG during the manufacturing process of baked goods has been studied. For instance, Hyvarinen et al (2006a) investigated the stability of SDG (derived from flaxseed) during baking of graham buns, rye breads and muffins. SDG remained stable during the baking process at 225 ^{o}C for 15 minutes. No significant change in SDG content was observed even after increasing both the baking temperature to 250 ^{o}C and the amount of time to 25 minutes. In the same study, SDG was found to be stable when stored at room temperature for 1 week and at freezing temperature for up to 2 months. Similar findings on SDG stability have been reported by Muir and Westcott (2000). Liukkonen et al (2003) also found that SDG was very stable during the fermentation and baking processes of sourdough rye bread production.

SDG stability in dairy products, such as milk, yogurt, cheese, and whey-based drinks also been evaluated. The SDG was stable throughout the dairy processing of high-temperature pasteurization, fermentation, and milk renneting (Hyvarinen et al 2006b). There was no significant change in the SDG content during the heating and fermentation of the milk. In addition, SDG stability was not affected by the addition of starter cultures (i.e. lactic acid bacteria and bifidobacteria) during the process of yogurt fermentation (Hyvarinen et al 2006b; Hall et al 2004). Similarly, the use of starter culture (i.e. lactic acid bacteria) and enzymes did not affect the stability of SDG in cheese manufacturing (Hyvarinen et al 2006b). However, 25 % of the added SDG was lost in the whey-based drinks when stored for 21 days at 4^{o}C. This loss was attributed to the low pH (3.0-4.1) of the whey drinks (Hyvarinen et al 2006b).

Thus far, most of the studies on lignan stability have been carried out on baked goods and dairy products, and little is known about SDG stability during other food processing techniques. Although Hyvarinen et al (2006b) analyzed the lignan stability in yogurt and cheese processing, no further studies have been conducted on other processed products, including sauerkraut, pickles, beer, and wine. However, Milder et al (2005) did evaluate the SECO content of these products (Table 2). Based on this work, the main conclusion was that these foods were not good sources of lignan. However, only SECO content was reported.

Table 2. Lignan (SECO) content (µg/100g) of selected fermented foods and beverages.

Fermented foods	SECO (µg/100g or µg/100ml)	Reference
Wheat Bread	15	Milder et al 2005
Beer (lager)	0-1.0	Milder et al 2005
Wine	5.2-61.3	Milder et al 2005
Grape Juice	10.8	Milder et al 2005

2.3.3. Other phytochemicals in flaxseed and their health implications

Although flaxseed is known for its lignan content, other phytochemicals, such as vitamins (e.g. folic acid) and phenolic acids (e.g. ferulic acid, gallic acid, m-coumaric acid, caffeic acid, and chlorogenic acid) exist in abundance (Liu 2013). In some cases, they are comparable to levels found in fruits. Fruits, in general, have the highest phenolic content, of which wild blueberries, blackberries, pomegranates, cranberries, and red grapes being the highest. These bioactive compounds are believed to be beneficial to health, for instance, reducing the risk of chronic diseases, such as cardiovascular disease and cancer. In the United States and other industrialized countries, cancer and cardiovascular diseases are two leading causes of death (Liu 2013).

2.3.3.1. Folates

Folate, a water soluble vitamin, is naturally found in foods or synthesized (e.g. folic acid). Both the natural folate and the synthetic form exist in the polyglutamate (long chain of glutamate molecules) and monoglutamate (one glutamate molecule) form, respectively (Koontz et al 2005). The structure of folate consists of pteroic acid and a side chain of conjugated glutamic acid molecules (Rampersaud et al 2003).

Folate deficiency, a well-recognized worldwide health problem, is linked with increased risk of cardiovascular diseases, megaloblastic anemia, and multiple birth defects, most notably neural tube defects (NTDs) (Blancquaert et al 2010). Globally, folate deficiency is responsible for approximately 300,000 to 400,000 children born with NTDs (spina bifida and anencephaly) every year (Williams et al 2002). In the US, an estimated 2500 pregnancies are affected with NTDs and in Europe, 4500 infants are born with this defect each year.

2.3.3.2. Phenolics

Phenolics, as mentioned earlier, are phytochemicals made up of one or more aromatic rings with one or two hydroxyl groups attached. The plant phenols have biological effects, such as antioxidant property (due to hydroxyl group on the structure), which is thought to have protective effect against cardiovascular disease. They lower oxidative stress that is caused by the saturation of highly reactive species in the body, including superoxide anion, hydrogen peroxide, and hydroxyl radicals (Dai and Mumper 2010). Oxidative stress is responsible for chronic degenerative diseases, such as heart disease, cancer, and aging. Phenolic compounds also inhibit the oxidation of important cell components, such as DNA, enzymes, lipids, and proteins (Dai and Mumper 2010). In an *in vitro* study, plant phenols prevented the oxidation of low-density lipoproteins (LDL), which is important because oxidized LDL are involved in the development of cardiovascular disease (Hollman 2001). Plant phenols have been recognized for their anti-carcinogenic effect. They are thought to have an inhibitory role in all stages of cancer: initiation, promotion, progression, and metastasis (Thompson 1998). The antioxidant behavior of phenols is hypothesized to be related to their ability to scavenge free radicals and possibly the prevention of cancer (Hollman 2001). Although health benefits of phenols have attracted the attention of medical researchers, the phenolic compound must be present in food after processing to be effective.

2.4. Overview of food processing

According to archaeological and ethnographic evidence, the hunter-gatherer societies utilized the first food processing methods (Fellows 2000). They used open fire heat and boiling water to prepare their meals, as well as increase palatability. Due to their nomadic life style, they did not need to preserve their food; however, after the invention of agriculture, societies started

to store and preserve their food. By 3000-1500 BC, the Egyptians started using the sun to dry/preserve fish and poultry, fermentation to produce alcohol, and cereal grinding to make leavened bread (Fellows 2000). As societies progressed, they started specializing in different food processing techniques, such as milling, baking, brewing, and cheese-making. Pastoral societies in the Middle East, also, started adopting similar techniques to prevent famine, improve their diet, and increase eating quality (Fellows 2000).

In those early days, the food processing techniques were simple craft skills that passed from one generation to another, and little to no effort was done to understand the science behind the processes (Fellows 2000). However, in the late 1700s, people with novel ideas started to be recognized, which lead to further discoveries. For instance, Napoleon Bonaparte awarded Nicholas Appert 12, 000 francs for discovering canning as a means to preserve foods for military and naval forces. Also, multiple food processing technologies we use today are products of World War I and World War II (Fellows 2000). Most food processing techniques, nowadays, are still used to increase shelf life, as well as enhance sensory characteristics of foods. In addition to the eating quality, current food industries aim to provide nutritional foods. Food products are being enriched with vitamins, minerals, and prebiotic cultures, resulting in functional foods. However, not all functional foods have been fully evaluated for retention of health promoting compounds.

2.4.1. Food processing effect on foods

2.4.1.1. Size reduction

There are desirable outcomes to food processing. For instance, size reduction (via milling) results in desirable textural and rheological properties, but can also result in unwanted aroma and flavor in some foods (Fellows 2000). By milling, one can improve mixing and heat

transfer, but such processing can disrupt cells and increase surface area, which in turn facilitates oxidative deterioration, and microbiological and enzymatic activities (Fellows 2000). For instance, oxidation of carotenes in flour can affect the color as well as the nutritional value. In some spices and nuts, increased temperature during milling can result in loss of volatile compounds (Fellows 2000). Erdman and Erdman (1982) reported a 78 % loss of Vitamin C in a sliced cucumber, which demonstrates an outcome of size reduction.

2.4.1.2. Mixing

Mixers, for blending foods or ingredients do not have any effect on nutritional value or shelf life. Mixing produces desirable sensory and functional properties. For example, the stretching and folding action during mixing results in gluten formation. Once formed, the strong structure of gluten gives breads the desired texture (Fellows 2000).

2.4.1.3. Heat processing

Heating is a very common food processing technique that improves eating quality (e.g. flavor). This technique is also used to preserve foods by inactivating enzymes and destroying microbes, insects, and parasites (Fellows 2000). Heating allows some foods to be shelf stable without refrigeration. Anti-nutrients such as trypsin inhibitors are destroyed by heating. It has been documented that heating increases availability of nutrients (e.g. niacin) and protein digestibility. On the other hand, heating can result in undesirable effects (e.g. flavor, color, texture, nutrient loss) by destroying food components.

Blanching is a heating method (pre-treatment) used to inactivate enzymes and remove air from foods (e.g. fruits and vegetables). This heat treatment has minimal effect on food quality as it utilizes a lower temperature (less than 100^0C) with a short time exposure. However, blanching

can result in the loss of some nutrients, including minerals and water-soluble vitamins (e.g. ascorbic acid) (Fellows 2000).

2.4.1.4. Pasteurization

This technique involves mild heat treatment (less than 100^0C) of foods. Pasteurization can inactivate enzymes and destroy both pathogenic and spoilage microbes; thus, extending the shelf life of foods, including bottled fruit and milk. This process is considered to have minimal effect on sensory characteristics and nutritional value. In pasteurized fruit juices, loss of vitamin C and carotene has been observed (Fellows 2000). In milk, loss of serum proteins and vitamin has been reported.

2.4.1.5. Sterilization

Sterilization is a heating process that involves the exposure of foods to high temperature for a longer time than blanching. This heating process is used to kill microorganisms and inactivate enzymes, extending the shelf life of foods. Unlike blanching, sterilization has detrimental effect on sensory characteristics and nutritional value of foods. For instance, canning, which utilizes the sterilization process, can promote hydrolysis of carbohydrates, proteins, and lipids. In canned meats, 10-20 % loss of amino acids has been observed (Fellows 2000). The loss of lysine increases with increasing heat treatment, maximum loss being 25 %. Further losses of two amino acids, methionine and tryptophan, have been shown to decrease the biological activity of protein up to 9 % (Fellows 2000). Vitamin loss also has been encountered during canning, particularly losses of thiamin (50 to 75 %) and pantothenic acid (20 to 35 %) have been reported. High loss of water-soluble vitamins such as ascorbic acid has also been recorded (Fellows 2000). On the other hand, sterilizing soy-meat increases nutritional value by inactivating trypsin inhibitors (Fellows 2000).

2.4.1.6. Fermentation

Fermentation is one of the oldest food processing techniques that has been practiced worldwide for millennia (Fellows 2000). Today, this technique is used to produce breads, alcoholic beverages, cheese, and other products. Unlike some food processing techniques, the mild condition of fermentation do not have detrimental effect on sensory characteristics and nutritional value (Fellows 2000). Mild changes such as protein and carbohydrate modification, during fermentation, brings about texture (i.e. softens) change to the final fermented product. By-products of fermentation (e.g. organic acids, ethanol) are also responsible for some flavor and aroma changes. On the other hand, microbial growth during the fermentation process can affect nutritive value (Fellows 2000). Microbes are able to alter the composition of proteins, fats, and carbohydrates. Microbes can also bring about a loss of nutrients because they are also capable of utilizing fatty acids, amino acids, sugars, and vitamins from foods (Fellows 2000). However, according to Dworschak (1982), there are nutritive microbes that are able to secrete vitamins. Microbes can also increase digestibility of proteins and polysaccharides by hydrolyzing polymeric compounds (Dworschak 1982).

2.4.1.6.1. Commonly fermented beverages

Two of the oldest fermented beverages include wine and beer. Wine is produced by fermenting grapes and other fruits. Beer, on the other hand, is commonly produced from barley malt, but it also can be produced from different types of starchy plants, including maize (e.g. South America), millet and sorghum (e.g. Africa), and rice (e.g. Far East) (FAO 2009).

Basic industrial brewing process comprises of mashing, boiling, and fermentation (FAO 2011). In the mashing step, hot water is used to extract soluble materials from grains (e.g. rice and barley) and sweet liquid called wort is produced in large wood/stainless steel vessels. The

wort is boiled and hops can be added for palatability and antiseptic purposes (FAO 2011). Before fermentation, wort must be cooled. Then, yeast is added into the wort and fermentation is carried out in large vats or food-grade plastic bins. The fermentation process can take up to two weeks depending on the storage temperature (FAO 2011).

Aside from beer production, use of barley for human consumption and the feed industry is increasing (Andersson et al 1999). The end quality of a product can be affected by protein content, lysine content, β-glucan, endosperm, and amylose contents of barley. Barley is comprised of starch (600 g/kg), total dietary fiber (200 g/kg), and protein (110 g/kg). The dietary fiber consists of β-glucan (30-70 g/kg) and arabinoxylans (40-70 g/kg), which are considered as important constituents (Andersson et al 1999). Other minor constituents in barley include fat (30 g/kg), ash (20 g/kg), and low molecular weight sugars (40 g/kg) (Aman et al 1985).

The carbohydrates and proteins in barley have been exhaustively studied (Niemi et al 2012). For example, the extraction of proteins and carbohydrates from brewer's spent grain (BSG-a major by-product of brewing) has been studied for the past 10 years (Niemi et al 2012). Complete removal of proteins and 50 % carbohydrate extraction from BSG has been achieved via protease activity and enzyme treatment, respectively (Niemi et al 2012). However, there has been a tremendous amount of work on barley phenolics, but very few on lignans. Smeds et al (2007) found that wheat had the highest lignan (SECO) amount (868 µg/100 g), followed by corn (125 µg/100 g), oat (90 µg/100 g), and barley (42 µg/100 g). Niemi et al (2012) determined a total of lignan content (1300 µg/100 g) in BSG, which was comparable to un-malted barley (Smeds et al 2007). Majority of these lignans were syringaresinol and secoisolariciresinol (SECO).

Wine production, on the other hand, involves fermentation of fruit juice with the addition of sugar and yeast (FAO 2011). First, fruit juice, yeast, and sugar are added into a fermentation bin for approximately ten days. During this stage, the bin is kept closed to avoid microbial contamination. After ten days, wine is filtered through a sterilized cloth and transferred into a narrow-necked fermentation vessel. The vessel is fitted with an air lock and fermentation is carried out at18 ^{0}C for approximately three weeks to three months (FAO 2011).

The source of phenolics in wine comes from the grapes or other fruits (e.g. blueberries) used for wine making (Recamales 2006). The presence of these phenolics in wine have good health benefits (i.e. free-radical scavenging and metal chelation), and contribute to the sensory characteristics, including color, flavor, and astringency (Lee and Jaworsky 1987). Due to the benefits associated with phenolics, studies have been conducted to access the stability of phenols during food processing. For instance, storing and aging wine affects polyphenol compounds, including anthocyanins, flavan-3-ol, and proanthocyanidins (Recamales 2006). These phenols have the tendency to react with other compounds, such as glyoxylic acid, pyruvic acid, and acetaldehyde, which results in pigment formation and ultimately wine color. Plus, proanthocyanidins have been reported to disappear during the color formation (Bakker and Timberlake 1997; Dallas et al 1996; Revilla et al 1999). Although similar studies have not been carried out on lignans during vinification, various lignan types have been quantified in white and red wines (e.g. secoisolariciresinol (7.6-61.3 µg/100 ml), matairesinol (2.7-7.8 µg/100 ml), lariciresinol (4.6-16.1 µg/100 ml), and pinoresinol (1.7-11.9 µg/100 ml) (Milder et al 2005).

As discussed earlier, the effect of food processing on SDG has been extensively studied in solid foods, including baked and dairy products, but little has been done on fermented beverages. The changes that occur as a result from food processing can be beneficial or

degradative. Understanding how processing affects food component is essential if functional foods are to be created. This thesis work addresses how processing affects lignan stability using processing methods associated with fermentation, as well as the SDG profile in foods with low-lignan content.

3. HYPOTHESIS

The fermentation process and the additives used during vinification, will not affect the amount of SDG. It is also expected for the other phytochemicals to remain constant during the mild process of fermentation. During aging/storage, both the amount of SDG and other phytochemicals is expected to remain unchanged. In addition, a significant difference in SDG content is expected among the barley varieties. An increase in SDG after the malting process is also expected. Finally, the amount of SDG is expected to decrease during the high heat treatments of the brewing process.

4. JUSTIFICATION AND OBJECTIVES

The incorporation of phytochemical-rich foods in our diet improves health (Blancquaert et al 2010; Liu 2013). However, to ensure their availability before consumption, one must understand the stability of these phytochemicals during processing treatments. Processing is known to change sensory and nutritional quality of foods (Fellows 2000). In addition, processing is known to affect the bioavailability of bioactive compounds (Fellows 2000). The effect of size-reduction, blanching, pasteurization, and sterilization on vitamins, proteins, fats, carbohydrates, and phytochemicals retention has been investigated in the past (Harper 1979; Dworschak 1982; Erdman and Erdman 1982; Fellows 2000). However, more research is needed on other processing methods, including malting, milling, vinification, and brewing. In this study, the stability of lignans (SDG) and other phytochemicals was assessed in a fermented juice process. The effect of malting and brewing on lignan (SDG) stability also was investigated. No data is available on the amount of SDG in barley; thus, the third objective was to determine the SDG content of different barley varieties.

5. MATERIALS AND METHODS

5.1. Materials

Golden flaxseed was obtained from Heartland Flax: Valley City, North Dakota. This flaxseed was used to prepare flaxseed extracts. Twenty Ethiopian and NDSU barley varieties (grown in Fargo, ND from 2012 and 2013) were provided by the NDSU Barley Varietal Development program. In addition, two malting grade barley samples were obtained from NDSU barley malt quality and pilot brewery laboratory: Conlon (2 row barley) and Robust (6 row barley). These samples were evaluated for lignans.

Welch's frozen grape juice (Welch's Food Inc. Concord, MA) and pure cane granulated sugar was obtained for a local grocery store. Lalvin K1-V1116 *Saccharomyces cerevisiae* (I.N.R.A –Montpellier. Lalvin, CA) and 1056 American Ale ™ yeast strain (Wyeast laboratories, Inc. Odell, OR) were used for the grape juice processing and brewing process, respectively.

Methyl alcohol (~99.9 %) and acetic acid (glacial, 99.85 %) were obtained from Sigma-Aldrich (St. Louis, MO). Secoisolariciresinol diglucoside (SDG) (~ 99.8 %) was obtained from ChromaDex (Irvine, CA). Folic acid (~97 %), ferulic acid (~99 %), gallic acid (~97 %), chlorogenic acid (~95 %), m-coumaric acid (~98 %) and caffeic acid (~98 %) were obtained from Sigma-Aldrich (St. Louis, MO).

5.2. Methods

5.2.1. Extraction of phytochemicals

Both flaxseed (i.e. golden flaxseed) and barley (i.e. different barley varieties, robust, and conlon barley varieties) were milled separately on a Z-mill (Retsch Inc.) using a 0.25 mm screen. Ground flaxseed was defatted with hexane using a Soxhlet extractor for 16 hours. Due to the low oil content in barley, no hexane extraction was carried out on the barley samples. Flaxseed (0.5

g) or barley (0.5 g) were placed into a screw-type test tubes (16 mm X 100 mm), followed by the addition of 10 ml of 70 % aqueous methanol. Test tubes were securely capped and vortexed for 30 seconds. Samples were incubated in a water bath for three hours at 60^0C. The test tubes were removed from the water bath every 15 minutes, and vortexed for 30 seconds during the one hour incubation. After the one hour incubation, test tubes were removed from the water bath and cooled using tap water. The test tubes were centrifuged (4100 rpm and 15^0C) for 20 minutes using a benchtop centrifuge. After centrifugation, 2 ml of supernatant was transferred into a clean screw cap test tube. The residue, after the centrifugation step, was discarded.

Into the test tube containing the 2 ml supernatant, 0.5 ml of 0.5 N sodium hydroxide was added and vortexed for 15 seconds. A color change from pale to bright yellow was observed. The sample was hydrolyzed for 3 hours at room temperature (22 ^{0}C). Afterwards, 0.5 ml of 0.5 N acetic acid was added to neutralize the sample. The neutralized extract (3 ml) was vortexed for 15 seconds and a color change from bright to pale yellow was observed. The neutralized extracts from the barley samples (i.e. barley extracts) were filtered through a 0.45 μm micro-filter (nylon acrodisc membrane) and transferred into HPLC vials. The neutralized extracts from the flaxseed samples (i.e. Flaxseed Extract) were saved and the extraction process was carried out repeatedly (30 times) until 90 ml of flaxseed extract was recovered for the grape juice fermentation study. Since there was a total of three replications of the grape juice fermentation study, the extraction process was carried out three times to produce a total of 270 ml. From the 90 ml FE obtained for each replication, only 85 ml was added into the grape juice solution. The other 5ml was retained as a control.

5.2.2. Analysis of phytochemicals

5.2.2.1. HPLC analysis

Lignans (SDG) and the other phytochemicals were analyzed using a Waters 2795 high performance liquid chromatograph (HPLC) attached to a Waters 996 Photodiode Array Detector (Mifford, MA). The HPLC was equipped with a LiChrosphere 100 RP- C18 column (5 µm, 250 mm × 4.5 mm). Column thermostat was set to 40 °C and injection volume was set to 10 µL. The mobile phase consisted of 1 % acetic acid (solvent A) and 100 % methanol (solvent B). Gradient conditions were as follows: A/B (v/v): 0 min (95:5), to 40:60 in 44 min, held 4 min, and back to 95:5 by 55 min. Lignans and the other phytochemicals were detected at 280 nm and peaks integrated with Waters Millennium Workstation Software (version 4.0). The SDG and the phytochemical peaks were confirmed and quantified by comparison with the appropriate standards. Linear HPLC calibration curves for standard SDG and the other phytochemicals were obtained for the concentrations of 0, 50, 500, 1000, and 2000 µg/ml (R value > 0.995). SDG was expressed in ug (SDG amount) per g (starting sample grain) for the barley samples. For the vinification and brewing process, the amount of phytochemicals (including SDG) were expressed in mg (phenolic compound amount) per ml (sample solution).

5.2.2.2. HPLC/TOF-MS analysis

Lignan analysis in barley was performed using an Agilent 6540 UHD Accurate –Mass Quadrupole Time-of-Flight mass spectrometer with AJS ESI source (Agilent Technologies, Santa Clara, CA, USA). The Agilent was connected to a UHPLC instrument (Agilent 1290 infinity) via an Electro Spray Ionization (ESI) source with Jetstream technology. MassHunter Quantification Analysis software was used to analyze chromatograms. Mass Spectra (ESI-MS) was acquired in the positive mode using the protonation molecule $[M\text{-}H]^+$. Nitrogen (N_2) was

used as the nebulizing and sheathing gas. ESI parameters included capillary voltage (4.0 KJ), flow rate (12 L/min), temperature (400 ^{0}C), nebulizer pressure (30 psi), fragmentor voltage (125 V), and mass analyzer scan range of (80 to 1100 (m/z). The UHPC conditions for the analysis consisted of the following: an Agilent Eclipse plus C_{18} RRHD column (2.1 mm × 50 mm, 1.8 µm) was used at 40 ^{0}C. Purine (1.0 ml) and HP-092 (0.8 ml) were used as reference standards for accurate mass reference. The mobile phase consisted of solvent A (0.1 % formic acid in high-purity water) and solvent B (0.1 % acetic acid in acetonitrile). The Gradient was set up for 13 minutes and was applied as follows: 0-2 min, 0-5 % B, 2-10 min, 5-95 % B, 10-12.50 min, 95 % B, 12.50-13 min, 95-10 % B. The flow rate was 0.4 ml/min and the injection volume was 10 µL. The SDG peak was analyzed using a Mass Hunter Workstation software (version B.05.00). For barley, standard solutions concentration range of 0, 0.01, 0.05, 0.5, and 1 mg/L were used to quantify SDG (R value > 0.995). Since there was no significant matrix effect on SDG content, the same calibration curve was used for raw and malted barley samples. In addition, the limit of detection (LoD) and limit of quantitation (LoQ) for SDG in the barley samples was 0.005 mg/L and 0.01 mg/L, respectively.

5.2.3. Grape juice processing (vinification)

The major steps carried out in the vinification process included primary fermentation, secondary fermentation, and aging. The procedure used for this study was adapted from Horn (1977). First, Welch's grape concentrate (340 ml) was transferred into a primary fermenting bucket and diluted to 3,785 ml (1 gallon). Into the grape solution, approximately 85 ml of the flaxseed extract (FE) was added. The initial specific gravity of the fortified grape solution with the FE was adjusted to 1.090 using granulated sugar (approximately 836 g). Then 0.3 g of potassium bisulfite was added into the fortified grape solution. Five samples (~ 1.5 ml) were

transferred into HPLC vials and saved for phytochemical analysis. The fortified grape solution was allowed to stand overnight in an incubator set at 22^0C. The following day, five samples (~ 1.5 ml) were transferred into HPLC vials and saved (@ 4^0C) for phytochemical analysis. Into the remaining fortified grape solution, 0.5 g of ammonium phosphate and 2.5 g of ammonium sulfate was added and stirred to dissolve all additives. After adding all the additives, the primary fermentation was carried out by sprinkling 0.5 g of yeast on top of the fortified grape solution. The bucket was loosely covered with a piece of cloth and put into an incubator (23^0C) for three days. On the third day, five samples (~ 1.5 ml) were transferred into HPLC vials and saved for phytochemical analysis. Lower specific gravity was obtained (~ 1.050), which indicated readiness for the secondary fermentation. After the end of primary fermentation, 200 ml of the fortified grape solution was siphoned from the fermenting bucket into five secondary fermenters (i.e. 250 ml Erlenmeyer flask). The fermenter was fitted with an airlock and was left in the incubator (23 ^{0}C) until the fermentation ceased. This secondary fermentation took approximately four weeks and every week, five samples (~ 1.5 ml) were transferred into HPLC vials and saved for phytochemical analysis. Once the fermentation was completed, no CO_2 bubble or belching through the fermentation lock was observed. Finally, the wine was aged in a dark place for 3 months at 22 ^{0}C. Every month, five samples (~ 1.5 ml) were transferred into HPLC vials and saved for phytochemical analysis (Figure 4). In addition, the pH of the grape solution was obtained at each step of the vinification process.

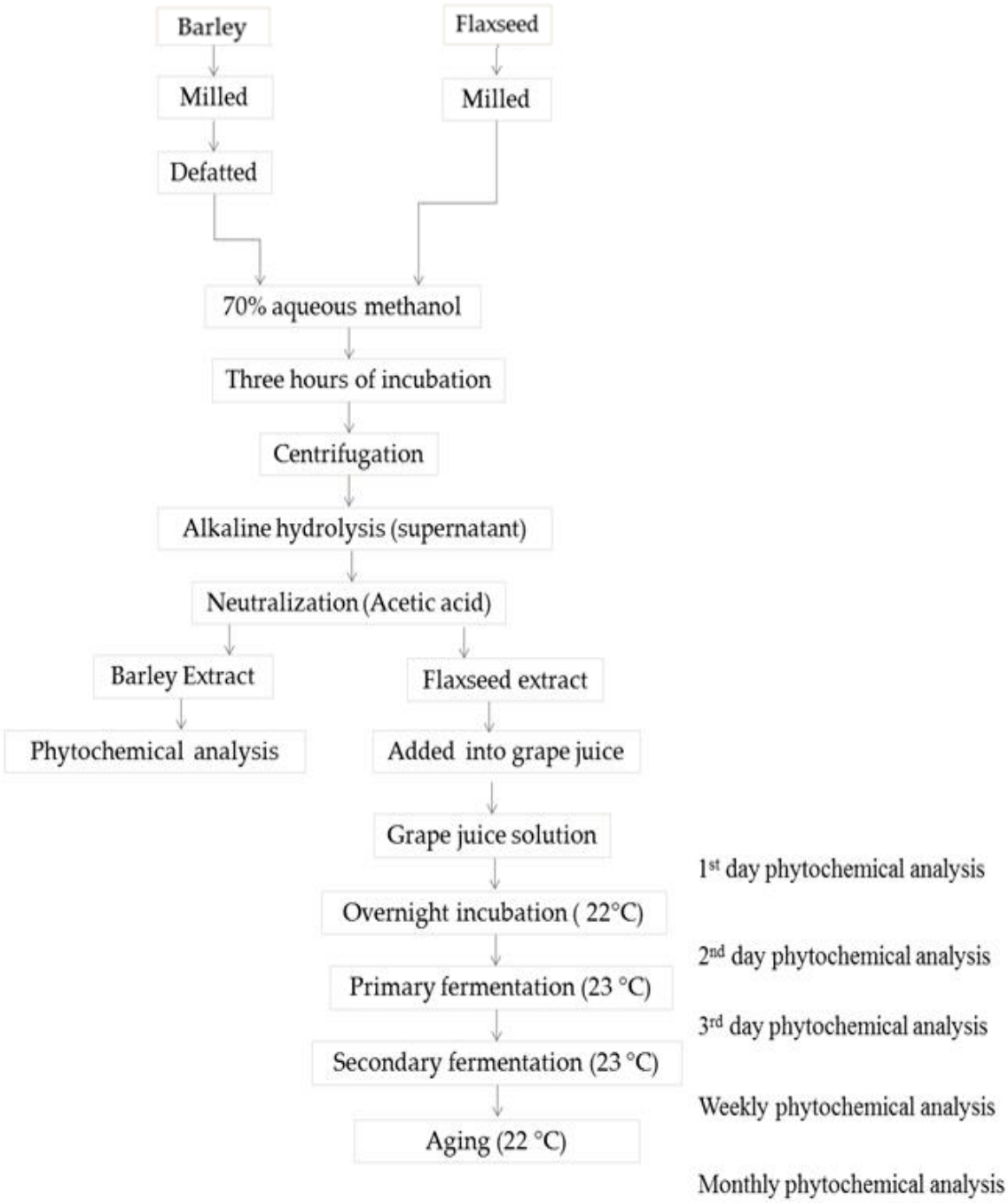


Figure 4. Flow chart of the phytochemical extraction, analysis, and vinification process.

5.2.4. Malting process

Malt was prepared from two different barley samples: Different barley varieties (grown in Fargo, ND from 2012 and 2013) and two malting grade barley: Conlon (2 row barley) and Robust (6 row barley). From the twenty barley varieties, ten were varieties used for food consumption, and the remaining ten were varieties used for brewing. For this study, the 20 barley varieties were only malted and not used for brewing. In contrast, the two malting grade barley varieties (i.e. Robust and Conlon) were used to prepare beer. Before malting, the steeping time was determined for all barley samples to attain 44 % moisture.

5.2.4.1. Steeping step

Twenty barley varieties (50 g) were placed in a 100 × 25 mm plastic centrifuge tubes. The centrifuge tubes had eight 3-mm holes to allow water flow. The test tubes were, then, placed into steeping baskets and the steeping process was carried out at 16 ^{0}C and six minute aeration with compressed air (Karababa et al 1993). The steep water was drained every 24 hour to allow air-rest of samples for 2 hours. Time of steeping for the twenty barley samples are shown below (Table 3). In addition, 300 g of robust and conlon barley samples were placed into different steep tank baskets. Similar steeping conditions as described above were followed (Table 4). Once steeped, barley samples were taken out of the steep tanks and spread over a paper towel for moisture removal.

Table 3. The twenty barley varieties and their steeping hours.

Location	Year	Variety	Barley Type	Steeping Time
Fargo	2012	Habesha Demoya-6R-W	Food	44
Fargo	2012	RAWSON	Brewing	50
Fargo	2012	Dimtu	Food	48
Fargo	2012	HB-120	Brewing	55
Fargo	2012	DESTA	Food	49
Fargo	2012	DIRBE	Food	48
Fargo	2012	ARUSO	Food	54
Fargo	2012	BEKA	Brewing	58
Fargo	2012	Agegnehu (218950-08)	Food	45
Fargo	2012	Estayish (218963-4)	Food	43
Fargo	2012	Bekoji-1	Brewing	48
Fargo	2012	2ND27421	Brewing	50
Fargo	2012	Abechu Demoye-6R-W	Food	39
Fargo	2012	2ND29835	Brewing	59
Fargo	2012	Netch gebs-6R-W-1	Food	44
Fargo	2012	ND26891	Brewing	52
Fargo	2012	Miscal-21	Brewing	54
Fargo	2012	HB-1307	Food	44
Fargo	2012	STELLAR-ND	Brewing	51
Fargo	2012	PINNACLE	Brewing	54
Fargo	2013	Agegnehu (218950-08)	Food	37
Fargo	2013	ND26891	Brewing	60
Fargo	2013	2ND29835	Brewing	48
Fargo	2013	2ND27421	Brewing	53
Fargo	2013	Abechu Demoye-6R-W	Food	34
Fargo	2013	RAWSON	Brewing	35
Fargo	2013	DESTA	Food	46
Fargo	2013	STELLAR-ND	Brewing	41
Fargo	2013	Netch gebs-6R-W-1	Food	35
Fargo	2013	Estayish (218963-4)	Food	37
Fargo	2013	BEKA	Brewing	45
Fargo	2013	PINNACLE	Brewing	44
Fargo	2013	HB-120	Brewing	38
Fargo	2013	ARUSO	Food	46
Fargo	2013	Bekoji-1	Brewing	37
Fargo	2013	Habesha Demoya-6R-W	Food	45
Fargo	2013	HB-1307	Food	44
Fargo	2013	Dimtu	Food	40
Fargo	2013	Miscal-21	Brewing	41
Fargo	2013	DIRBE	Food	44

Table 4. Two barley varieties (i.e. Robust and Conlon) used for studying the effect of brewing on the SDG content.

Barley Varieties	Barley Type	Steeping Time (Hour)
Conlon	Brewing	55.09
Robust	Brewing	55.38

5.2.4.2. Germination step

5.2.4.2.1. Germinative Energy

Kernels (100) of each barley sample were transferred into two glass petri dishes (90 mm) containing filter papers (Whatman no.1) for germinative energy determination. Once the kernels were uniformly distributed within the petri dishes, 4 ml of distilled water was added and covered with a lid. All closed petri dishes were packed in a polyethylene bag and placed in a dark cabinet (20 ^{0}C). Sprouted kernels were removed from each petri dish after 24, 48, and 72 hours. Using the formula below, germinative energy (GE) for each barley was calculated.

$$GE\ (\%) = \frac{\text{(Sum of sprouted kernels in the two petri dishes after 72 hours)}}{2}$$

This step was only used to test the malting quality of the grain. For all barley samples, the GE (%) was above 95 %.

Then, all the barley samples from the steeping step were transferred into 400 ml beaker and placed into a germination cabinet. The germination was carried out for four days at 16^{0}C with a relative humidity of 95 % (Karababa et al 1993).

5.2.4.3. Kilning step

All germinated barley samples were placed in kiln baskets and dried over a temperature range of 49 to 85 ^{0}C for 24 hours (Karababa et al 1993). Once dried, the rootlets from the barley

samples were removed and malted barley was obtained. The malted samples (5 g) were saved for lignan analysis.

5.2.5. Brewing process

Cream Ale (an American-styled brew) was used for this study. The brewing process was carried out on the NDSU 4.0 L laboratory brewery (Figueroa et al 1987). The brewing process included a cleaning, mashing, Lautering, boiling, and fermentation step. Samples were taken from each step of the brewing process.

5.2.5.1. Cleaning, grinding, and ingredients

Before brewing, the pilot brewery lines, vessels, and kettle were cleaned with hot water. In addition, the pilot brewery was cleaned with a Star San sanitizer (1 oz. /5 gallons of tap water). The main ingredients for the brewing process included two types of malted barley, namely conlon (two-row malt barley) and robust (six-row malt barley). Two adjuncts also were used, including pre-gelatinized flaked corn and rahr two-row. All ingredients, except for the flaked corn were ground using Buhler-Miag laboratory malt mill and saved for lignan analysis (~ 5 g). In addition, the brewing process was carried out twice using different amounts of ingredients. The first brewing process was comprised of four ingredients: conlon (2.3665 kg), robust (0.9589 kg), flaked corn (0.9845 kg), and rahr two-row (0.4615 kg). The second brewing process comprised conlon (0.9412 kg), robust (1.4378 kg), flaked corn (0.9841 kg), and rahr two-row (0.9226 kg). These ingredients were subjected to mashing.

5.2.5.2. Mashing

All four ingredients from the first and second brewing process were added to 12 L of warm (68 ^{0}C) water while stirring. After the addition of the malt, the temperature was lowered to 63 ^{0}C and the mashing was carried out for 45 minutes. Then, iodine drops were applied on

mashed samples every 10 minutes to check the conversion of starch into sugars. Once the conversion was achieved (i.e. color change from yellow to amber), the sample was held in the mash tank for an additional ten minutes at 75 ^{0}C. The total mash time was 94 minutes. Then, mashed samples (~ 5 g) were saved for lignan analysis. The remaining mash was moved to a lauter tun.

5.2.5.3. Lautering

After mashing, 8 L of 75^{0}C sparge water was transferred from a hot liquor tank into the lauter tun containing the mash sample. After 15 minutes, the mash solution was recirculated to separate the liquid portion from the solid. Once the liquid (wort) cleared, it was transferred into a brew kettle. Approximately 27 L of wort was transferred into the brew kettle. Wort sample (2 ml) was saved for lignan analysis.

5.2.5.4. Wort boiling

The remaining wort (~ 27 L) was heated to boiling for 10 minutes and hops were added. The boiling was carried out for additional 60 minutes and boiled wort was transferred into a whirlpool. Hops (~31 g) were added for aroma and the whirlpool was allowed to cool for 10 minutes. Boiled wort (2 ml) was saved for lignan analysis and the remaining wort was moved to the fermentation process.

5.2.5.5. Fermentation

Approximately, 20 L of wort was transferred into a carboy and carbonated with oxygen. Then, yeast (Wyeast 1056 American Ale) was added and fermentation was carried out at 20 ^{0}C for four weeks. Final beer samples (2ml) were saved for lignan analysis.

5.2.6. Statistical Analysis

Data were subjected to analysis of variance (ANOVA) to determine significant differences among treatments (food processing times) using SAS Software (version 9.3, SAS Institute Inc., Cary, NC). Differences were considered to be significant at p values ≤ 0.05. All data were reported as means ± SD (standard deviation) on an as is basis.

5.2.6.1. Randomized Complete Block Design (RCBD)

A split-plot in time principle (repeated measure analysis) was applied to the wine and beer preparation study, where successive analysis was made on the same experimental unit over a period of time during the vinification and brewing process. The main plot being the experimental unit (i.e. fermentation bucket containing the beverages) and the subplot being the repeated measurements at different times (i.e. samples subjected to lignan and other phytochemical analysis). However, RCBD design was implemented since the repeated measure analysis requires at least two factors as opposed to one factor (i.e. one type of grape juice), which was used in our study. Thus, for the RCBD analysis, the vinification process was replicated three times and the brewing process was replicated two times. In addition, the different condition at different time of the food processing step was considered as the treatment.

The RCBD design was also used for comparing the lignan (SDG) content of the twenty barley varieties from the two years (2012 and 2013). Treatments included variety, barley type (food or brewing), and malted barley. The two years were considered as two replications. In addition, log transformation (LSDG) was carried out to minimize the wide variation of SDG content in the barley samples.

6. RESULTS AND DISCUSSION

6.1. Stability of phytochemicals during vinification process

6.1.1. Effect of vinification on lignans (SDG)

The stability of SDG was determined after subjecting grape solution, containing FE, through fermentation and aging (Table 5). Three replicates were carried out to determine the SDG level, but only two replicates were used. The SDG levels in the third replicate had similar increasing trend throughout the aging like the other two replicates, but the SDG content was very high (results not shown) and was considered as an outlier. One explanation for this high SDG level could be the amount of FE added at the initial step of the vinification process. Another explanation could be due to the SDG extraction procedure, or more specifically during the alkaline hydrolysis step. Depending on the hydrolysis time and degree of hydrolysis, the amounts of SDG extracted can vary.

Significant differences were observed in the levels of SDG at different times of the vinification process (Table 5). The FE-fortified grape solution during the first 10 days had a constant SDG level. However, the amount of SDG increased by 16 % during the three fermentation weeks (Table 5). The SDG content increased further during the last three months of aging. Overall, the SDG level increased by 30 % during the vinification process (Table 5).

Table 5. Secoisolariciresinol diglucoside (SDG) level (µg/ml) during the vinification process.

Dates of sampling	SDG level (µg/ml)-fermentation
Day 1 (grape juice solution + FE)	67.24 ± 0.001 a
Day 2 (overnight incubation)	67.46 ± 0.002 a
Day 3 (1st day of fermentation)	65.22 ± 0.003 a
Day 10 (1st week of fermentation)	66.50 ± 0.002 a
Day 17 (2nd week of fermentation)	76.50 ± 0.002 b
Day 24 (3rd week of fermentation)	75.88 ± 0.002 b
Day 31 (4th week of fermentation)	78.25 ± 0.002 b
Day 61 (1st month of aging)	83.98 ± 0.001 c
Day 91 (2nd month of aging)	87.73 ± 0.001 c
Day 121 (3rd month of aging)	87.41 ± 0.002 c

Means in a column with the same letter are not significantly different at $p < 0.05$; n= 2
Values are expressed as mean ± SD

6.1.2. Effect of vinification on other phytochemicals

The stability of other phytochemicals also was determined after subjecting grape solution, containing FE, through fermentation and aging. Statistically, the processing condition had significant effect on some of the phytochemicals (Table A.1). Gallic acid level remained unchanged until after day 2, and then increased 26 % between days 3 and 10 (Figure 5a). The gallic acid level, then, decreased by 25 % during days 17 and 61. By the end of the vinification process (Day 121), the gallic acid level had increased by 45 % (Figure 5a). Similarly, the caffeic acid level remained unchanged until the first week of fermentation (Day 10) (Figure 5b).

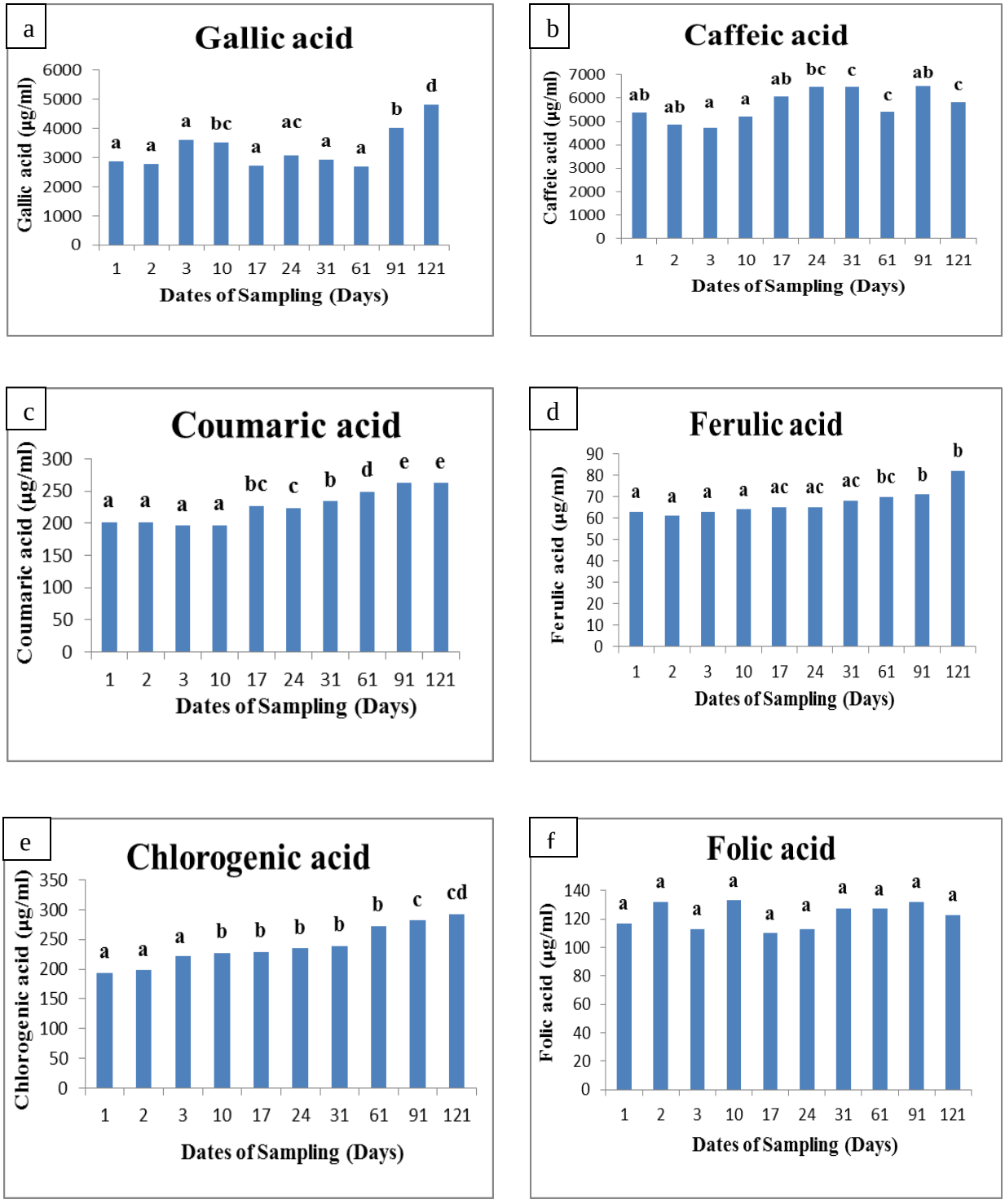


Figure 5. Gallic acid (a), caffeic acid (b), coumaric acid (c), ferulic acid (d), chlorogenic acid (e), and folic acid (f) level (µg/ml) during the vinification process. Means with the same letter are not significantly different at $p < 0.05$; n= 3.

Although a clear trend was not obtained, about 24 % increase in caffeic acid was observed after the second week of fermentation (Day 17) (Figure 5b).

The amount of coumaric acid remained unchanged until the first week of fermentation (Day 10) of the vinification process (Figure 5c). However, coumaric level increased by 15 % during days 17 and 31. Coumaric level continued to increase until the end of the aging process (Day 121) (Figure 5c).

Ferulic acid remained unchanged until the third week of fermentation (Day 24), but increased by 26 % afterwards (Figure 5d). Similarly, chlorogenic acid remained stable until day 2 and increased by 17 % afterwards (Figure 5e). The chlorogenic acid level was stable during days 3 and 31, but increased by 23 % afterwards until the end of the aging process (Day 121) (Figure 5e). In addition, the amount of folic acid remained stable throughout the vinification process (Figure 5f).

The pH at different steps of the vinification process remained relatively stable (Table 6). A slight increase in acidic pH, during the vinification process, was observed. This suggests that FE did not affect the fermentation.

Table 6. pH values at each step of the vinification process.

Vinification steps	pH
Grape juice solution	3.3
End of primary fermentation	3.1
End of secondary fermentation	3.1
End of Aging	3.1

6.2. Discussion on phytochemicals during food processing

6.2.1. Structural changes of polyphenols

Food processing and storage are thought to alter the chemical structure of soy isoflavones, polyphenols that have similar chemical structure to lignans (Shimoni 2004). For example, soy isoflavones mainly exist as glycosides (i.e. phenols with sugar molecules attached), similar to lignans and studies have shown some change to these glycosides during food processing (Shimoni 2004). For instance, Wang and Murphy (1994) determined high level of glycosylated soy isoflavones in non-fermented foods and high level of aglycones (i.e. phenols without sugar molecule) in fermented foods. In our study, however, the amount of SDG did not decrease; it either increased or remained stable throughout the fermentation and aging process. The other phytochemicals had similar stability and incremental increases during the vinification process. An explanation for the increase could be due to the release of SDG and the other phytochemicals from the FE. In other words, if the FE complex was not completely hydrolyzed during the alkaline hydrolysis, the remaining phytochemicals might have been released during the vinification process, due to hydrolysis of the FE complex during the fermentation and aging steps.

6.2.2. Oxidative browning effect on phytochemicals

In wine, phenols, more specifically phenols with two hydroxyls attached (i.e. o-diphenols), are more susceptible to oxidative browning. Both enzymatic as well as non-enzymatic reactions are responsible for the browning reactions (Cheynier et al 2000). The enzymatic browning occurs only in the grape must and the non-enzymatic reaction occurs both in the grape must and in the wine (Cheynier et al 2000).

During grape processing, the intact cells of grape tissues get disrupted and phenols get exposed to atmospheric oxygen and oxidoreductase enzymes, including peroxidase (POD) and polyphenol oxidase (PPO). Once exposed, the phenols get oxidized and cause browning (Li et al 2008). The iron-containing enzyme (POD) has minimal effect on phenols, but if present with PPO, it can degrade phenols (Li et al 2008). On the other hand, the copper-containing enzyme, PPO, is known to oxidize phenols, more specifically, mono-phenols and o-diphenols. For instance in grape must, PPO oxidizes caftaric acid and p-coumaric acid into a powerful oxidant called caffeoyltartaric acid o-quinones (CTAQ), which are known to oxidize other compounds in wine that bring about sensory changes (Robards et al 1999). First, mono-phenols get hydroxylated into o-diphenols and diphenols, in the presence of PPO and oxygen. Further oxidations of diphenols produce o-quinones that react with amino acids and hydroquinones, resulting in brown pigments (Robards et al 1999). However, such enzymatic oxidation during red wine processing is limited. Similarly, no degradation of phenols was observed in our study. In addition, the relatively acidic pH (Table 6) during the vinification process had minimal effect on phenols.

On the other hand, non-enzymatic browning occurs in the absence of PPO after the fermentation of wines (Li et al 2008). Phenols can oxidize in the presence of air, high temperatures, high pH, sunlight, and metal ions (Fe and Cu). O-diphenols (e.g. caffeic and gallic acid) are, for instance, very susceptible to non-enzymatic oxidation (Li et al 2008). However, in our study, no phenol degradation has been observed, suggesting the absence of oxidation.

6.2.3. Folates

Similar increases in food components, during food processing, have also been observed in various studies (Katina et al 2007; Liukkonen et al 2003). For example, folates and phenolic

compounds increased during baking with rye. The yeast used during this sourdough fermentation process was identified as the cause for the increase (Kariluoto et al 2004). The addition of yeast (i.e. *Saccharomyces cerevisiae*) during the sourdough fermentation increased the folate level by 54%. In contrast, the amount of folates remained unchanged during similar sourdough fermentation without yeast. Osseyi et al (2001) reported a 73 % increase in folate levels during the production of wheat bread. The presence of yeast during the baking process was a major factor for the increase in folate content. Seyoum and Selhub (1998) reported that baker's yeast was found to contain folates (>2000 µg/100g), which can explain the increased folate amount during the baking process. Additional researchers have confirmed the increase in the folate level in wheat breads, and that approximately 53–65 % of the folates come from the yeasts used during the bread baking (Butterfield and Calloway 1972; Keagy et al 1975). In our study, however, the amount of folates remained unchanged throughout the vinification process.

6.2.4. Enzymes role during food processing

The increase of SDG and other phenolics might be due to the release of phenols from bound phenolics during fermentation. For instance, according to Abdel –Aal and Rabalski (2013), the amount of free ferulic acids increased by 17 % and bound phenolics decreased by 36 % during the production of wholegrain breads. Similar increase in phenols has been reported by Gelinas and McKinnon (2006) during the production of wholegrain breads. In the current study, all phenolic acids and SDG increased during fermentation. This increase in phenolic acids could be due to the release of bound phenols during the fermentation and aging process of wine. Similar findings have also been reported by Budic-Leto and Lovric (2002) during the fermentation and aging of white wines. They observed increased levels of phenolic acids, such as vanillic, caffeic, p-coumaric, and ferulic acids. The increase likely resulted from the hydrolysis

of ester bound phenols like hydroxycinnamic acids (HCA) (e.g. caftaric, coutaric, fertaric) during fermentation (Budic-Leto and Lovric 2002).

SDG is not found in its free form, but in complex with other SDG residues, coumaric acid glucoside (CouAG), and ferulic acid glucoside (FeAG). The release of SDG and phenolics from these complexes cannot fully explain the increase in SDG level (30 %) and other phytochemicals (up to 45 %) during the vinification process. Another explanation for this apparent increase in SDG could be due to the feruloyl esterases found in the wine yeast. Brewer's yeast is known to contain these esterases that can cleave ester bonds between plant cell wall polysaccharides and phenolic acids (Benoit et al 2006). During the vinification process, any remaining bound-SDG via ester linkage could have been cleaved by these enzymes, releasing free SDG as well as phenolic acids.

Various researchers have documented the stability of SDG during the baking process and storage (Hyvarinen et al 2006a; Muir and Westcott 2000; Liukkonen et al 2003). However, according to a lignan stability study conducted on rye, the total amount of lignans increased by two-fold and three-fold after germination and fermentation, respectively (Katina et al 2007). These two food processing techniques comprise the hydration of grains at a certain condition to activate both endogenous and added enzymes, which in turn can bring about changes, including structure, bioactivity, flavor, stability, and digestibility. Liukkonen et al (2003) reported the increase (2─3.5 folds) of folates and methanol-soluble phenolic acids (e.g. lignans) during a germination period of 6 days at 15─25 ^{0}C. Katina et al (2007) stated that the increase during germination and fermentation was due to the synthesis and action of hydrolytic enzymes, which in turn can activate reactions and aid in the synthesis of new compounds. In addition, the outer layer of rye kernel contains endogenous enzymes and indigenous microbes that can alter grain

composition when activated (Katina et al 2007; Loponen et al 2004). Thus, in our study, the increase in most of the phenols, including SDG, gallic acid, caffeic acid, coumaric acid, chlorogenic acid, and ferulic acid may have been due to yeast enzyme activity during the vinification process.

The effect of enzymes on chemical compounds has been documented mostly in a solid matrix, such as the rye kernel. Nogueira et al (2008) investigated the effect of alcoholic fermentation (cider processing) on phenolic compounds. For instance, the amount of total phenols remained unchanged in three cider apple varieties and decreased in two varieties of the cider. An increase in caffeic acid and catechin content, regardless of the cider varieties also was observed (Nogueira et al 2008). Apart from the enzyme effect, no clear explanation or mechanism was proposed for the synthesis of these phenolic compounds. However, few researchers have proposed possible explanation for the degradation of phenolic compounds (Siebert et al 1996; Renard et al 2001; Guyot et al., 2003). For instance, the maceration step in cider processing enhances the activity of polyphenol oxidase (PPO), which results in oxidation of polyphenols (Nogueira et al 2008). In addition, the change in procyanidin during the production of French ciders has been observed (Alonso-Salces et al 2004). Some of these reactive properties included the binding of procyanidin with proteins and cell wall polysaccharides (Alonso-Salces et al 2004). As a result, a major reduction in procyanidin has been reported during cider processing (Alonso-Salces et al 2004). This scenario was not likely a reason for the increased phenolic content obtained in our study, but provides a possible reason for increasing phenolic compounds during food processing.

6.3. Stability of phytochemicals during malting and brewing process

6.3.1. SDG analysis in different barley (raw) varieties

The SDG analysis for the 20 barley varieties (grown in Fargo, ND from 2012 and 2013) are presented on page 49 (Table 7). SDG content was determined in the barley varieties (Appendix Figure B.2). Unlike the vinification process, the amount of SDG in these barley varieties was very small, thus measured in µg/g.

The highest SDG content in the harvest year 2012 occurred in the food variety Dirbe (25.79 µg/g) and the lowest occurred in another food variety Desta (0.01 µg/g) (Table 7). In the harvest year 2013, Estayish (food variety) had the highest SDG level (13.04 µg/g) and HB-120 (brewing variety) had the lowest SDG level (0.10 µg/g) (Table 7). In contrast, six barley varieties had non-detectible SDG content in the year 2012. In 2013, five barley varieties had non-detectible concentration of SDG (Table 7; Appendix Figure B.3).

In addition, the year-to-year variation in SDG content was different among varieties. For example, the SDG content in Rawson was 0.13 µg/g in 2012 and 2.37 µg/g in 2013. In contrast, the SDG content in Aruso was higher (0.52 µg/g) in 2012 compared to 0.20 µg/g in 2013. This year-to-year variation could be explained by the different growing conditions (e.g. climate and soil type) in 2012 and 2013.

Table 7. Secoisolariciresinol diglucoside (SDG) content (µg/g) in twenty barley varieties grown in Fargo, ND.

Variety (raw)	Barley Type	SDG content (µg/g)	
		2012	2013
Habesha Demoya-6RW	Food	0.15	nd
Rawson	Food	0.13	2.37
Dimtu	Food	0.17	nd
HB-120	Brewing	3.74	0.10
Desta	Food	0.01	nd
Dirbe	Food	25.79	nd
Aruso	Food	0.52	0.20
Beka	Brewing	nd	nd
Agegnehu (218950-08)	Food	0.11	0.45
Estayish (218963-4)	Food	0.08	13.04
Bekoji-1	Brewing	nd	0.51
2ND27421	Brewing	nd	0.46
Abechu Demoye-6RW	Food	nd	0.19
2ND29835	Brewing	0.12	3.94
Netch gebs-6R-W-1	Food	0.27	0.11
ND26891	Brewing	0.72	0.38
Miscal-21	Brewing	3.06	0.22
HB-1307	Food	0.10	4.38
Stellar-ND	Brewing	nd	0.27
Pinnacle	Brewing	nd	0.38

Note: (nd) none detected indicates that SDG concentration was lower than detection limit (0.005 mg/L).

Only 10 barley varieties had detectible SDG content in both 2012 and 2013 (Table 8). From these varieties, Estayish (218963-4) and Netch gebs-6R-W-1, both food varieties, contained the highest (6.56 ± 9.17 µg/g) and lowest (0.19 ± 0.11 µg/g) SDG concentrations, respectively (Table 8). The two years (2012 and 2013) were considered as replications and

accounted for substantial variations in SDG concentrations. Due to this variation, no significant differences between the 12 barley varieties were found (Appendix Table A.2). To minimize this variation, the SDG value was transformed into logarithm and was subjected to statistical analysis, but no significant differences were observed (data not shown).

6.3.2. Analysis of food and brewing barley varieties for SDG content

The amount of SDG was compared in the food and brewing barley varieties. The average SDG content in the food barley (1.93 ± 2.73 µg/g) was higher than the brewing barley (1.48 ± 0.60 µg/g) (Table 8). However, the large standard deviation resulted in a CV of 41% for the brewing varieties and 141 % for the food varieties.

Table 8. Mean values of SDG content (µg/g) of raw barley varieties from year 2012 and 2013.

Variety (raw)	Barley Type	SDG content (µg/g)
Agegnehu	Food	0.28 ± 0.24
Aruso	Food	0.36 ± 0.23
Estayish (218963-4)	Food	6.56 ± 9.17
Netch gebs-6R-W-1	Food	0.19 ± 0.11
HB-1307	Food	2.24 ± 3.03
	Food barley Average	1.93 ± 2.73
Rawson	Brewing	1.25 ± 10.13
ND26891	Brewing	0.55 ± 0.24
Miscal-21	Brewing	1.64 ± 2.01
HB-120	Brewing	1.92 ± 2.58
2ND29835	Brewing	2.03 ± 2.71
	Brewing barley Average	1.48 ± 0.60

Values are expressed as mean ± SD, n=2

6.3.3. Effect of malting on SDG content

The raw barley varieties discussed above were malted and the SDG level was determined (Table 9). The highest SDG content in the harvest year 2012 occurred in the malted food variety-

Habesha Demoya-6RW (6.50 µg/g) and the lowest occurred in the malted brewing variety-Bekoji-1 (0.04 µg/g) (Table 9). In the harvest year 2013, HB-120 (brewing variety) had the highest SDG level (16.02 µg/g) and pinnacle (brewing variety) had the lowest SDG level (0.02 µg/g) (Table 9). In contrast, 11 barley varieties had non-detectible SDG content in the year 2012. In 2013, five barley varieties had non-detectible concentration of SDG.

Table 9. The SDG content (µg/g) in twenty barley varieties after malting.

Variety (raw)	Barley Type	SDG content (µg/g)	
		2012	2013
Habesha Demoya-6RW	Food	6.50	nd
Rawson	Food	0.07	nd
Dimtu	Food	nd	nd
HB-120	Brewing	0.60	16.02
Desta	Food	nd	0.28
Dirbe	Food	1.34	0.32
Aruso	Food	nd	0.42
Beka	Brewing	4.04	0.24
Agegnehu (218950-08)	Food	0.26	0.41
Estayish (218963-4)	Food	nd	nd
Bekoji-1	Brewing	0.04	nd
2ND27421	Brewing	nd	0.66
Abechu Demoye-6RW	Food	nd	0.29
2ND29835	Brewing	0.10	0.40
Netch gebs-6R-W-1	Food	nd	5.76
ND26891	Brewing	nd	0.46
Miscal-21	Brewing	nd	1.66
HB-1307	Food	0.35	0.55
Stellar-ND	Brewing	nd	2.98
Pinnacle	Brewing	nd	0.02

Note: (nd) none detected indicates that SDG concentration was lower than detection limit (0.005 mg/L).

Only 6 malted barley varieties had detectible SDG content in 2012 and 2013 (Table 10). Statistically, there was no significant difference among the malted samples (Appendix Table A.3). But, the SDG content varied widely in the malted barley samples. From these varieties, HB-120 (brewing barley) and 2ND29835 (brewing barley) contained the highest (8.31 ± 10.90 µg/g) and the lowest (0.25 ± 0.21 µg/g) SDG level, respectively (Table 10).

6.3.4. Analysis of malted food and brewing barley varieties for lignan (SDG) content

The amount of SDG was compared in the food and brewing malted-barley varieties. The average SDG content in the food barley (0.54 ± 0.26 µg/g) was lower than the brewing barley (3.57 ± 4.22 µg/g) (Table 10). However, the large standard deviation resulted in a CV of 118% for the brewing varieties and 48 % for the food varieties.

Table 10. Mean SDG content (µg/g) of barley varieties after malting.

Variety (malt)	Barley Variety	SDG level (µg/g)
HB-1307	Food	0.45 ± 0.14
Dirbe	Food	0.83 ± 0.72
Agegnehu (218950-08)	Food	0.33 ± 0.10
	Food barley average	0.54 ± 0.26
HB-120	Brewing	8.31 ± 10.90
Beka	Brewing	2.14 ± 2.69
2ND29835	Brewing	0.25 ± 0.21
	Brewing barley average	3.57 ± 4.22

Values are expressed as mean ± SD, n=2

6.3.5. Difference between raw and malted barley

The SDG content in the raw and malted barleys were compared (Table 11). Statistically, no significant difference was observed between the raw and malted barley samples (Appendix Table A.4). However, differences in the SDG content were observed. The average SDG content

of the raw barley varieties (2.08 ± 0.23 µg/g) was lower than the malted barley varieties (4.38 ± 5.56 µg/g). The CV for the raw and malted barley varieties are 11 % and 127 %, respectively. The large % CV supports the non-significance between raw and malted barley samples.

Table 11. Secoisolariciresinol diglucoside (SDG) content (µg/g) in raw and malted barley varieties from 2012 and 2013.

Barley variety	SDG content (µg/g)
HB-120 (R)	1.92 ± 2.58
HB-1307 (R)	2.24 ± 3.03
Average	2.08 ± 0.23
HB-120 (M)	8.31 ± 10.90
HB-1307 (M)	0.45 ± 0.14
Average	4.38 ± 5.56

Values are expressed as mean ± SD, n=2
Note: (R) denotes raw barley and (M) denotes malted barley

6.3.6. Effect of brewing on SDG

The SDG content was determined for all the ingredients used in the brewing process (Table 12). SDG was detected both in Robust and Conlon barley varieties. SDG was not detected in the flaked corn and in Rahr two-row barley. The corn used for the brewing process was not malted, so no data was obtained for the malt ingredient (Table 12). Rahr two-row barley was already a malted ingredient, thus no data was obtained for the raw ingredient.

Table 12. SDG levels (µg/g) of brewing ingredients.

Brewing ingredients	SDG level (µg/g) (RAW)	SDG level (µg/g) (MALT)
Robust	0.09	0.07
Conlon	0.11	0.09
Flaked corn	nd	na
Rahr two-row	na	nd

Note: (nd) none detected, (na) not available.

Using these ingredients, two brewing processes were carried out to study the effect of brewing (i.e. mashing, lautering, wort boiling, and fermentation) on SDG levels (Table 13). During the mashing step, no SDG was detected in the solid portion (spent grain). Similarly, during the lautering step, no SDG was detected in the liquid portion (wort). No SDG was detected during the wort boiling and fermentation step of the brewing process (Table 13).

Table 13. SDG levels (µg/g) during the brewing process.

Brewing process	Mashing	Lautering	Wort boiling	Fermentation
1st rep	nd	nd	nd	nd
2nd rep	nd	nd	nd	nd

Note: (nd) none detected

6.4. Discussion on composition and stability of SDG during food processing

6.4.1. Composition of SDG in barley varieties

In this barley study, the aim was to investigate the effect of variety, malting, and brewing on lignan (SDG) content. The SDG values showed high variability between replicates. However, the values obtained from this study can be used as an indicative rather than a definitive result. No barley SDG data has been reported in the past. Very few studies have been conducted on barley lignans and if conducted, only lignan SECO (the aglycone) has been determined in barley and some other foods (Milder et al 2005; Mazur et al 1996; Adlercreutz and Mazur 1997; Mazur 1998; Muir and Westcott 2000).

One explanation for the high % CV between the samples could be attributed to the barley samples used for the study. The same barley varieties from two years (2012 and 2013) were considered as two replicates, which may not have been sufficient. Another explanation for the variability could be a difference in the growing condition /environment of the barley varieties in those two years. Environmental factors affect on the phenolic content in plants is well

documented (Ali and Abbas 2003). In addition, the statistical analysis did not show significant differences among barley varieties, but the amount of SDG varied greatly in the ten barley varieties analyzed (Table 8). Studies have also shown the influence of variety on phenolic compounds (Maillard et al 1996; Holtekjolen et al 2006).

In this study, the SDG content in barley was very small. Compared to oil seeds, cereals have very low lignan (SECO) content, which supports the low values obtained from this study. According to Smeds et al (2007), the SECO content for the following cereals is as follows: rye (462 µg/100 g), wheat (868 µg/100 g), corn (125 µg/100 g), and barley (42 µg/100 g). Unlike flaxseed, barley is saturated with carbohydrates (78─83 %) that can affect the extraction of phenols from the food matrix. One explanation for the low lignan content, in our study, could be due to the extraction procedure. In addition, known amount of SDG was spiked into raw and malted barley samples; the SDG amount remained unchanged, indicating no matrix effect. No methods were available for extracting SDG from cereals; thus, the same extraction procedure used for flaxseed was used to extract SDG from the barley samples (method section in this paper). On the other hand, many methods were available for extracting the aglycone form of the lignan (SECO) and other phenolics (Smeds et al 2007; Milder et al 2004; Milder et al 2005). For extracting the aglycone (SECO), acid (hot hydrochloric acid) or enzyme (β-glucosidase) hydrolysis must be carried out after organic solvent extraction and alkaline hydrolysis, respectively (Milder et al 2004; Liggins et al 2000). However, the main target in this study was to analyze the SDG content, and it was possible to determine the SDG content in barley using the extraction method of Westcott and Muir (1996). To my knowledge, no data is available on SDG content in barley, thus this study can be used as a stepping stone for future studies.

6.4.2. Stability of SDG during malting and brewing

Other than variety, processing affects the content of polyphenols in barley (Goupy et al 1999). For instance, malted barleys had lower polyphenol content than their corresponding raw barleys (Goupy et al 1999). However, in this study, the amount of SDG in the raw barley was lower than the malted barley (Table 14). Similarly, Katina et al (2007) reported an increase in lignan content by two-fold during the germination of wholemeal rye. In their study, the processing condition was similar to an industrial malting of barley. In contrast, the fermentation of the germinated wholemeal rye did not affect the lignan content, but increased the content of phenols nearly 11 fold (Katina et al 2007). This increase was attributed to the starter culture (*Saccharomyces cerevisiae*) used during the fermentation process (Katina et al 2007). Although the exact mechanism is not clearly known, enzymes from microbes and outer layers of the cereal enhance hydrolytic enzyme activities, which are thought to alter grain composition (Katina et al 2007; Loponen et al 2004). Some of these enzymes include amylases, xylanases, and proteases. In addition, fermentation aids in the breakdown of cell walls, resulting in the liberation or synthesis of functional compounds (Katina et al 2007).

During the brewing process, no SDG content was observed. The mashing step, a relatively mild process, which involves the soaking of malt barley in hot water (68-75 ^{0}C) cannot explain the disappearance of SDG. During the SDG analysis, representative sample may not have been taken from the mash tun, which could explain the SDG absence during that step. SDG was not detected in the solid and liquid portion of the mashed sample. In addition, it is hard to obtain representative sample from big experimental units (i.e. mashing tun, lauter tun, brewing kettle, and carboy). For the remaining steps of the brewing process, similar absence of SDG was obtained. To obtain definitive results, the brewing process must be done in a smaller scale.

7. CONCLUSION

The hypothesis of this research was that the phytochemicals remain constant during the vinification process. Therefore, we reject the hypothesis because a 30 % increase in SDG during the vinification process was observed. Similarly, the concentration of the other phenolic compounds (i.e. gallic acid, chlorogenic acid, coumaric acid, caffeic acid, and ferulic acid) increased. However, folic acid remained unchanged during the vinification process. In addition, SDG content was, for the first time, determined in barley. Statistically, no significant differences were obtained among barley varieties; however, variations in SDG content among barley varieties were observed, specifically the year-to-year variation. As hypothesized, the SDG content was affected by the mashing, lautering, boiling, and fermentation treatments of the brewing process. Overall, the processing conditions used in our study brought changes to the composition of phytochemicals.

8. FUTURE WORK

Although the results obtained in this study have demonstrated the stability of lignans and other phytochemicals during vinification, an in-depth study is required with the microbes used during the fermentation process. From this study it is not possible to predict exactly what caused the phytochemical content to increase. This increase could be attributed to the *Saccharomyces cerevisiae* used during the process, but to verify that, other microbes used during food and beverage fermentation should be tested. It is also important to test the synergistic effect of two or more microbes on phytochemicals.

Furthermore, extraction methods for foods with low lignan content must be improved. In this study, an extraction method used for flaxseed-SDG was used to obtain SDG from barley. However, cereal grains have a different food matrix than oilseeds; thus, the amount of SDG might be underestimated. Therefore, it is recommended to develop a specific extraction method for SDG in barley.

Finally, the experimental design used for the barley varieties should be improved. The use of years (2012 and 2013) as replications introduced large variability to the SDG values in the barley cultivars. To minimize such variability, it is recommended to compare SDG levels in barley cultivars grown in the same year and environment. Using more replicates can also strengthen the experimental design and produce definitive results.

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APPENDIX A. STATISTICAL ANALYSIS OF PHYTOCHEMICALS DURING THE VINIFICATION, MALTING AND BREWING PROCESS

Table A.1. ANOVA of the phytochemicals content during the vinification process.

Dependent variable	Food processing dates F-value	DF	P-value
SDG	17.01	9	0.0001
Gallic acid	15.70	9	0.0001
Caffeic acid	5.11	9	0.0016
Ferulic acid	17.01	9	0.0001
Coumaric acid	97.35	9	0.0001
Chlorogenic acid	28.10	9	0.0001
Folic acid	0.52	9	0.8423 (N.S)

Table A.2. ANOVA of SDG in 12 raw barley varieties.

Dependent variable	Raw barley varieties F-value	DF	P-value
SDG	0.73	11	0.6981

Table A.3. ANOVA of SDG in 6 malted barley varieties.

Dependent variable	Malted barley varieties F-value	DF	P-value
SDG	0.32	5	0.8807

Table A.4. ANOVA of SDG content in raw vs. malted barley.

Dependent variable	Raw vs. malted barley varieties F-value	DF	P-value
SDG	0.89	7	0.5577

APPENDIX B. HPLC AND LC-MS CHROMATOGRAPHS OF PHYTOCHEMICALS

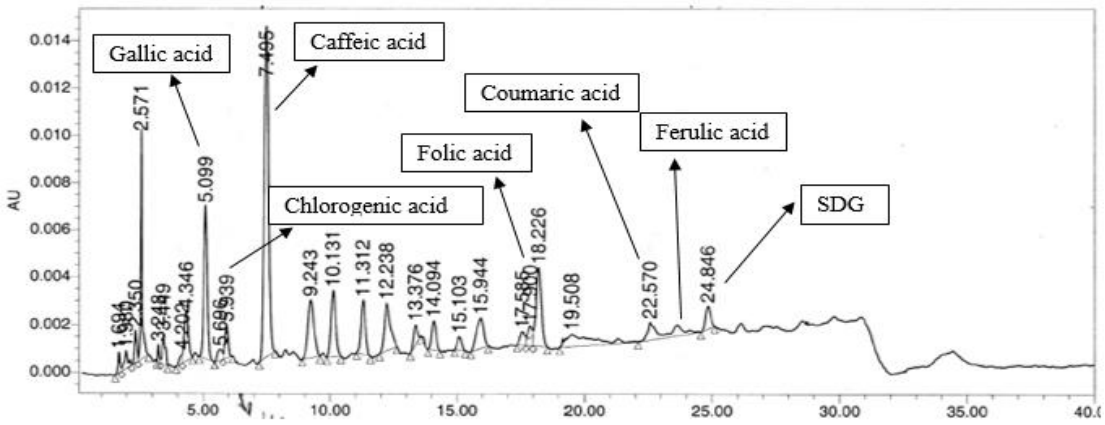

Figure B.1. HPLC chromatograph of phytochemicals during the vinification process.

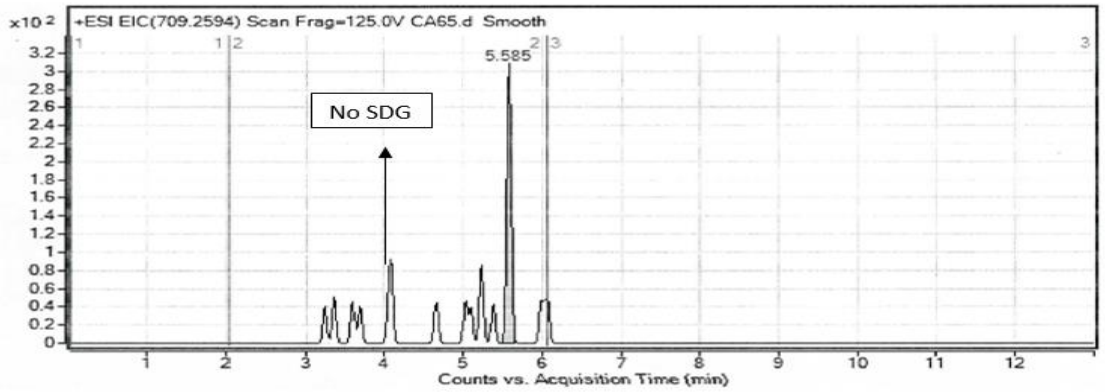

Figure B.2. LC-MS chromatograph of barley extract with non-detectible SDG.

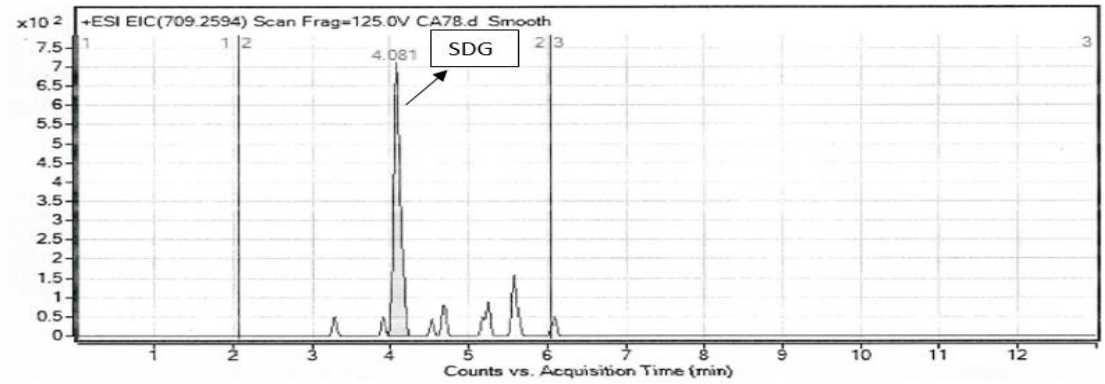

Figure B.3. LC-MS chromatograph of barley extract with detectible SDG.